ECOLOGY OF BACTERIA FROM ANTARCTIC HYPERSALINE LAKES.

by

SIMON R. JAMES, B.Sc. (Hons), Tas.

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ABSTRACT

There exists a unique and characteristic bacterial biota in Antarctic hypersaline lakes and 15 new bacterial species have been described from the lakes of the Vestfold Hills, Antarctica. In this study, antibodies were raised against the type strains of the four bacterial species which have been most consistently isolated from the oxylimnion of the hypersaline lakes of the Vestfold Hills. The abundance of these serogroups was measured throughout the austral spring and summer in four hypersaline lakes and a seawater site by indirect immunofluorescence microscopy. Maximum abundances of *Halomonas* serogroups (up to 40% of the total bacteria) and the *Flavobacterium gondwanense* serogroup (up to 10% of the total bacteria) were observed at discrete depths within the water column in the two most hypersaline lakes at about midsummer, coincident with the time of maximum sunlight and the commencement of the summer thaw. Change in species abundance was not reflected in the total bacterial count, indicating compositional change of the total bacterial population.

Concurrently, radioisotope incorporation methods were used to estimate heterotrophic bacterial productivity and metabolism. In the coastal marine site, incorporation rates were within the range previously reported for Antarctic ecosystems, ranging up to 2.1 pM thymidine h^{-1} and 11.0 pM leucine h^{-1}. Incorporation rates in the hypersaline lakes ranged up to 4.6 pM thymidine h^{-1} and 94 pM leucine h^{-1}. Generation times throughout all sample sites were fastest after the summer thaw at a depth of 2 m. Even at these peak times productivity in the hypersaline lakes was more than 100 times lower than average productivity in temperate aquatic environments. Optimum temperature for radioisotope incorporation was close to in-situ temperature in most cases, confirming the adaption of in-situ bacteria to the low water temperatures.

Dissolved organic carbon (DOC) concentrations reflected the strong stratification and seasonal variation of other lake parameters. The ratio of [^{3}H]leucine incorporation to [^{3}H]thymidine incorporation (a measure of bacterial physiological state or species composition) correlated with DOC concentrations. DOC concentrations correlated well with total bacterial numbers but not with changes in species composition.

In the meromictic hypersaline lakes, reduction of sulphur compounds is also an important means of energy generation. The *Halomonas* and *Flavobacterium* species isolated so far do not appear to be involved in sulphur cycling. However, a novel species of *Brevibacterium*, isolated and partly described in this study, reduces dimethylsulfoxide to dimethylsulphide and utilises methionine and cysteine as sole energy sources.

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LIST OF ABBREVIATIONS

Genus abbreviations

H.	*Halomonas*
D.	*Deleya*
F.	*Flavobacterium*
B.	*Brevibacterium*

Other abbreviations

ACAM	Australian Collection of Antarctic Micro-organisms
a_w	water activity
CLM	chemiluminescence
CPM	counts per minute
DAPI	4,6-diamidino-2-phenylindole
DFAA	dissolved free amino acids
DM_2S	dimethyl disulphide
DMS	dimethyl sulphide
DMSO	dimethyl sulphoxide
DOC	dissolved organic carbon
DPM	disintegrations per minute
ESR	external standard ratio
FDC	frequency of dividing cells
FITC	fluoroscein isothiocyanate
FPD	freezing point depression
GC-MS	gas chromotography/mass spectrophotometry
GF/C	Whatmann GF/C filter (nominal 2-4 μm pore size)
HPLC	high pressure liquid chromotography
Leu	L-[3,4,5-^{3}H]leucine
PCR	polymerase chain reaction
pH_{min}	nominal minimum pH for growth
pH_{opt}	optimum pH for growth
ratio of Leu:Tdr	ratio of Leu incorporation to Tdr incorporation
$Salts_{max}$	nominal maximum %NaCl for growth
$Salts_{opt}$	optimum %NaCl for growth
SCR	single channel ratio
SWS	sea water salts
TBZ	temperature below zero
Tdr	[*methyl*-^{3}H]thymidine

TMA*N*-O	trimethylamine *N*-oxide
T_{max}	notional maximum temperature for growth
T_{min}	notional minimum temperature for growth
T_{opt}	optimum temperature for growth

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Non-refereed publications

James, S.R. 1991. Ecology of Antarctic micro-organisms. Annual report to the Antarctic Division of The Department of Science (Available from Antarctic Division, Kingston, Tasmania).

James, S.R. 1991. New tools for Microbial Ecology. Australian National Antarctic Research Expeditions Newsletter (ANARE news). Kingston, Tasmania.

Refereed publications

James, S.R., Burton, H.R., McMeekin, T.A. & Mancuso, C.A. 1994. Seasonal abundance of *Halomonas meridiana*, *Halomonas subglaciescola*, *Flavobacterium gondwanense* and *Flavobacterium salegens* in four Antarctic lakes. *Antarctic Science*, **6**:325-332.

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1. INTRODUCTION

This study was undertaken to increase understanding of the microbial activity in the hypersaline lakes of the Vestfold Hills. Data was collected from a range of environmental and biological conditions during 1990/91 and supplemented with laboratory analysis of samples returned to the University of Tasmania. The particular focus of this study is the identification of species distribution, determination of whole community activity within the lakes and the correlation of these phenomenon to variation in seasonal conditions.

The existence of a distinctive bacterial microbiota in Antarctic hypersaline lakes has been firmly established though only a small part of these populations has been described. The Vestfold Hills, on the coast of Antarctica, is an ice free area of approximately 400 km^2. Distributed in these Hills are more than 60 hypersaline lakes and several hundred fresh water lakes and ponds. New species of bacteria have been readily isolated from these hypersaline lakes (Franzmann & Dobson, 1993; Hirsch & Siebert, 1991b). These species are tolerant to a broad range of salinity and temperature (Franzmann & Dobson,1993).

The hypersaline lakes of the Vestfold Hills are catchment relicts which contain seawater isolated by isostatic uplift in the last 6000 years (Burton, 1981). The bacterial biota of these lakes appears to have been of marine origin as most culturable species from the oxylimnion of the hypersaline lakes of the Vestfold Hills are closely related to marine bacteria (Dobson *et al.*, 1993; Franzmann & Tindall, 1990). Growth and metabolism of halotolerant bacteria in Antarctic sea ice microbial communities occurs at $\geq$ -1.9 °C and $\leq$ 90 ‰ salts. Some organisms from this community may well have colonised the hypersaline Antarctic lakes (Kottmeier & Sullivan, 1988).

The lakes studied were meromictic; permanently stratified with lighter, less saline water overlying a more saline layer. This permanent stratification leads to anoxia in the lower layer caused by bacterial respiration over long periods of time. Meromictic lakes are not common worldwide, but 28 have been characterised from the Vestfold Hills (J. Gibson, Pers. Comm., 1995).

Descriptions of the changing microbial environment and the associated changes in bacterial number and state have limited use unless many of the species involved can be recognised and enumerated; an understanding of the microbial ecology of an environment requires studies of temporal and spatial changes in species composition (Brock, 1987; Tabor & Neihof, 1982). Four bacterial species have been consistently isolated from the oxylimnion of the meromictic hypersaline lakes of the Vestfold Hills. These were *Halomonas meridiana, H. subglaciescola, Flavobacterium gondwanense* and *F. salegens.* In this study immunofluorescence microscopy was used to enumerate the spatial and temporal change of these target species in specific Antarctic environments. The technique has been used extensively in medical microbiology (Herbert, 1990) for the detection of microbial pathogens (Kaspar & Tartera, 1990) and to a lesser extent in the field of microbial ecology (Dahle & Laake, 1982; Herbert, 1990; Reed & Dugan, 1978; Ward & Perry, 1980; Xu *et al.,* 1984). The seasonal abundance of these species was quantified throughout the austral spring and summer in the meromictic Organic Lake, Ekho Lake, Fletcher Lake and Ace Lake and a local seawater site. Six other lakes were sampled once during summer: Horse (now Williams) Lake, Johnston Lake, Straight-In (now Burch) Lake, Laternula Lake, Cemetery Lake and Deep Lake (Figure 1.1; Table 1.1).

Concurrent with this immunological investigation, the spatial and temporal variation in bacterial activity in the meromictic hypersaline lakes was examined by incorporation of both [^{3}H]thymidine and [^{3}H]leucine.

Once DNA synthesis is initiated within the bacterial cell it proceeds at a fixed rate to completion. Further, the synthesis of DNA does not occur in non-growing cells (Moriarty, 1986). While rates of synthesis of RNA and protein vary with nutritional status and growth rate (Staley & Konopka, 1985), DNA synthesis is directly associated with cell division. Thymidine is taken into the dividing cell and used almost exclusively as a precursor to DNA synthesis (Moriarty, 1990). Most heterotrophic marine bacteria can utilise thymidine in this way (Fuhrman & Azam, 1982). By radiolabelling the thymidine molecule, the rate of DNA synthesis (and hence productivity) can be ascertained. Measurement of the productivity of the whole heterotrophic population can be complemented by the measurement of rates of metabolic activity by the

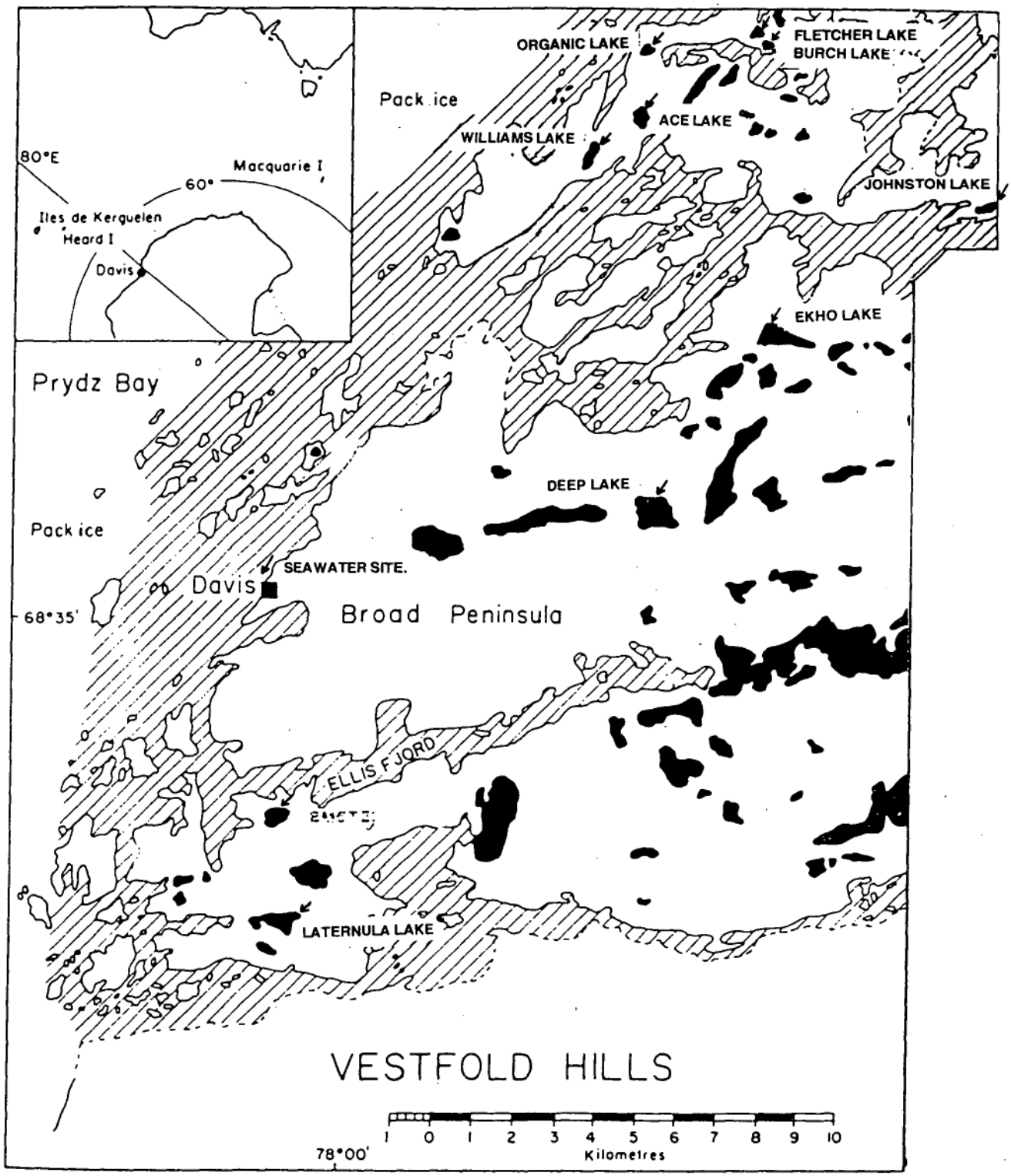


Figure 1.1: Map of the Vestfold Hills, Antarctica, showing location of sample sites. Map modified from Franzmann *et al.* (1988a).

Table 1.1: Location and characteristics of lake and seawater sites sampled during 1990-1991.

Site	Salinity range[a] (‰)	Temperature range[b] (°C)	Lake depth (m)	Depth of oxycline (m)
Organic Lake (68°27.2'S.,78°12.3'E.)	66 to 215	-10 to +8	7	4
Ekho Lake (68°31.2'S.,78°16.0'E.)	35 to 166	-3 to +19	42	20
Fletcher Lake (68°27.2'S.,78°15.2'E.)	25 to 126	-5 to +8	12	8
Ace Lake (68°28.4'S.,78°11.1'E.)	10 to 51	-3 to +11	23	11
Local sea water (68°34.1'S.,77°59.0'E.)	33 to 35	-2 to -1	3	NA
Burch Lake (68°27.4'S.,78°15.7'E.)	145 to 172	-5 to +9	6	—[c]
Laturnula Lake (68°39.0'S.,77°58.0'E.)	180 to 198	-8 to +8	6	—[c]
Cemetery Lake (68°37.5'S.,77°59.6'E.)	201 to 222	-11 to +8	1	—[c]
Deep Lake (68°33.6'S.,78°11.6'E.)	210 to 280	—[c]	36	—[c]

[a] Lake salinity range from a summer time surface melt-water minimum to constant maximum levels in the anaerobic bottom waters.

[b] Lake temperatures range from a winter upper waters minimum to a summer maximum in the middle waters.

[c] Data not collected.

incorporation of radiolabelled leucine into protein. As experiments are incubated for only a short time, radiolabel incorporation measurements can be taken without changing the rates of DNA or protein synthesis.

Bacterial production has been examined by radioisotope incorporation in many different environments. The results obtained in this study were compared with data obtained in Arctic and Antarctic marine ecosystems (Bailiff *et al.*, 1987; Bunch & Harland, 1990; Kottmeier & Sullivan, 1988), temperate hypersaline environments (Oren, 1990; Tibbles *et al.*, 1992) and Antarctic marine hypersaline environments (Kottmeier & Sullivan, 1988).

Background environmental variables such as temperature, depth, density (at 20°C) and dissolved organic carbon content were routinely measured at each sample site (Table 1.2). Bacteria from many of the sample sites were examined for the ability to reduce sulphur compounds as this is an important part of the ecology of many of the lakes (Franzmann *et al.*, 1988a). From these isolates a novel species of the genus *Brevibacterium* was partly characterised.

Table 1.2: List of environmental variables determined for each water sample as well as minimum and maximum values over the entire data set.

Variable	Units	Range
Day	days from 1/12/89	0 to 428
Depth	metres	0 to 40
Temperature	degrees celsius	-11.0 to 19.0
Salinity	parts per thousand	0 to 280
Chlorophyll	grams per litre	0.0 to 190.0
[Oxygen]	ml per litre	0.0 to 16.0
pH	pH	7.0 to 8.6
Bacterial Numbers	cells per ml	4.0×10^5 to 9.0×10^7
%H. meridiana	% of total numbers	0 to 23
%H. subglaciescola	% of total numbers	0 to 20
%F. gondwanense	% of total numbers	0 to 12
%F. salegens	% of total numbers	0 to 4
unfiltered DOC	mg per litre	0.0 to 80.0
1.0μm filtered DOC	mg per litre	0.0 to 100.0
0.6μm filtered DOC	mg per litre	0.0 to 110.0
0.4μm filtered DOC	mg per litre	0.0 to 75.0
0.2μm filtered DOC	mg per litre	0.0 to 45.0
Productivity - Tdr	pM per hour	0.01 to 5.00
Protein synthesis - Leu	pM per hour	0.02 to 90.00
Ice cover	metres	0.0 to 2.0
Snow cover	millimetres	0 to 250

2. LITERATURE REVIEW

2.1 The Vestfold Hills, Antarctica.

Since 1981 a number of biogeochemical processes occurring in the lakes of the Vestfold Hills, Antarctica, have been examined in detail. These include sulfur cycling (Franzmann *et al.*, 1987b; Franzmann *et al.*, 1988a; Gibson *et al.*, 1991; Hand & Burton, 1981), bacterial photosynthesis (Burke & Burton, 1988), bacterial temperature response (McMeekin & Franzmann, 1988), methane generation (Franzmann *et al.*, 1991b) and the distribution and abundance of volatile organic molecules (Roberts & Burton, 1993). Volkman *et al.* (1988) and Mancuso *et al.* (1990) examined microbial community structure by analysis of microbial pigments and lipids. Microorganisms involved in these processes, including several new species (Dobson *et al.*, 1991; Franzmann & Rohde, 1991; Franzmann *et al.*, 1987a; Franzmann *et al.*, 1988b; Franzmann *et al.*, 1991a; James *et al.*, 1990; McGuire *et al.*, 1987), have been isolated and characterised from the lakes of the Vestfold Hills and the associated local marine areas.

The Vestfold Hills area has the highest concentration of meromictic lakes anywhere in the world (Burton, 1981a). Salinity, temperature and other physical parameters within the water column show greater variation than most other aquatic environments. The lakes of the Vestfold Hills have been investigated for a variety of reasons; the unique nature of the robust and tolerant endemic microbiota, the extraordinary chemical processes demonstrated within the lakes and the uniqueness of the external environment. These lakes offer a compelling environment for study.

2.1.1 Meromictic lakes

Hand & Burton (1981) report that meromictic lakes are rare throughout the world; only 150 were known in 1975. However in Antarctica, meromictic lakes occur in the Dry Valleys, Penguin Island, Lutzow-Holm Bay, Deception Island and the Vestfold Hills. The latter area has at least 28 meromictic lakes (J. Gibson, Pers. Comm., 1995) within 400 km^2.

The hypersaline lakes of the Vestfold Hills have a salt composition derived from sea water (thalassohaline). Stratification seems to have developed as the local climate shifted and the lake levels rose and fell (J. Gibson, Pers. Comm., 1995). Compounding this, seasonal freezing exudes brine which, being heavier than the normal lake water, collects in the depths of the lake. Summer thaw and fresh water inflow dilute the upper few meters of the lake. Stratification persists as the lakes are protected from wind mixing by a semi-permanent ice cover (Gallagher *et al.*, 1989). Bacterial activity over long periods of time leads to anoxia in the bottom waters which are almost isothermal. The mixolimnion is well oxygenated and exhibits a seasonal thermal regime. The abrupt density increase of the various haloclines can act as one-way mirrors, trapping sunlight and warming particular strata in the lake.

The presence of clines, particularly haloclines or oxyclines (boundary layer between aerobic and anaerobic lake water masses), can have a dramatic effect on the structure of a microbial community, leading to the development of strong vertical stratification.

2.1.2 Microbiota of the lakes of the Vestfold Hills

The terms psychrotolerant and halotolerant are used throughout this review as general descriptors of bacteria which grow well over a wide range of temperature or salinity. Those bacteria growing obligately below 20 °C or above 3% salts are termed psychrophiles (after Morita, 1975) or halophiles (after "moderate-extreme halophiles", Kushner, 1978) respectively.

Bacteria from low-nutrient environments are more nutritionally versatile and grow over a wider variety of physiological conditions than bacteria isolated from high-nutrient environments (Horowitz *et al.*, 1983). This seems to apply to bacteria from environments where harsh conditions render many of the nutrients refractory. Most isolates from the Vestfold Hills lakes can grow on a wide range of single carbon sources and the majority of strains collected from the lakes of the Vestfold Hills are halotolerant and psychrotolerant (Mancuso *et al.*, 1991).

The majority of isolates from the lakes of the Vestfold Hills grow optimally at temperatures above 20 °C but are capable of growing to 0 °C. The majority of these isolates have diverged from their nearest

know relatives at least 200 million years ago (estimated from 16S rRNA dissimilarity; Franzmann & Dobson, 1993). Bacteria have opportunistically colonised the Vestfold Hills area from unknown sources.

2.1.3 Potential application of Antarctic microbial research

Baseline studies are needed in pristine Antarctic environments to help determine the impact of any future human activity. A study by Ellis-Evans *et al.* (1994) on the impact of human habitation on an Antarctic lake concluded that: "...proximity to a research station has rapidly influenced a simple continental Antarctic lake system...".

From a more pragmatic viewpoint, the salt lake environment is a potential source of useful bioactive compounds and bacterial diversity. Extreme environments have long been considered potential reservoirs of bacteria able to produce useful bioactive compounds. Microscopy by Hirsch & Siebert (1991a) showed visible evidence of biodiversity (Figure 2.1). From the abundance of morphotypes observed compared to the few isolated, it is obvious that many more species exist within the lake system than have so far been described.

Ashbolt (1990) summarised areas of potential biotechnological application of microorganisms from the hypersaline lakes. Extremophiles, particularly thermophiles and halophiles, are of interest to scientists in the biotechnology industry. The enzyme systems and metabolic processes of bacteria adapted to low temperature and high salinity may be exploited in low-energy biochemical processes. Antarctica offers a huge diversity of unique psychrotolerant and halotolerant microorganisms (Ashbolt, 1990; McMeekin *et al.*, 1993).

Bacteria in most communities experience regular diurnal or seasonal changes. In upwelling regions, estuaries, littoral zones and sea-ice habitats, bacteria may experience rapid changes in temperature or salinity. Localised short term (decade long) episodic changes occur in continental water bodies. These changes vary the physical condition and distribution of microbial communities (Hawes *et al.*, 1994). Jones and Ellis-Evans (1994) indicate that the physiochemical parameters of several lakes studied in the Signy Islands were highly variable within the Holocene period. Dramatic short term changes in sediment and nutrient

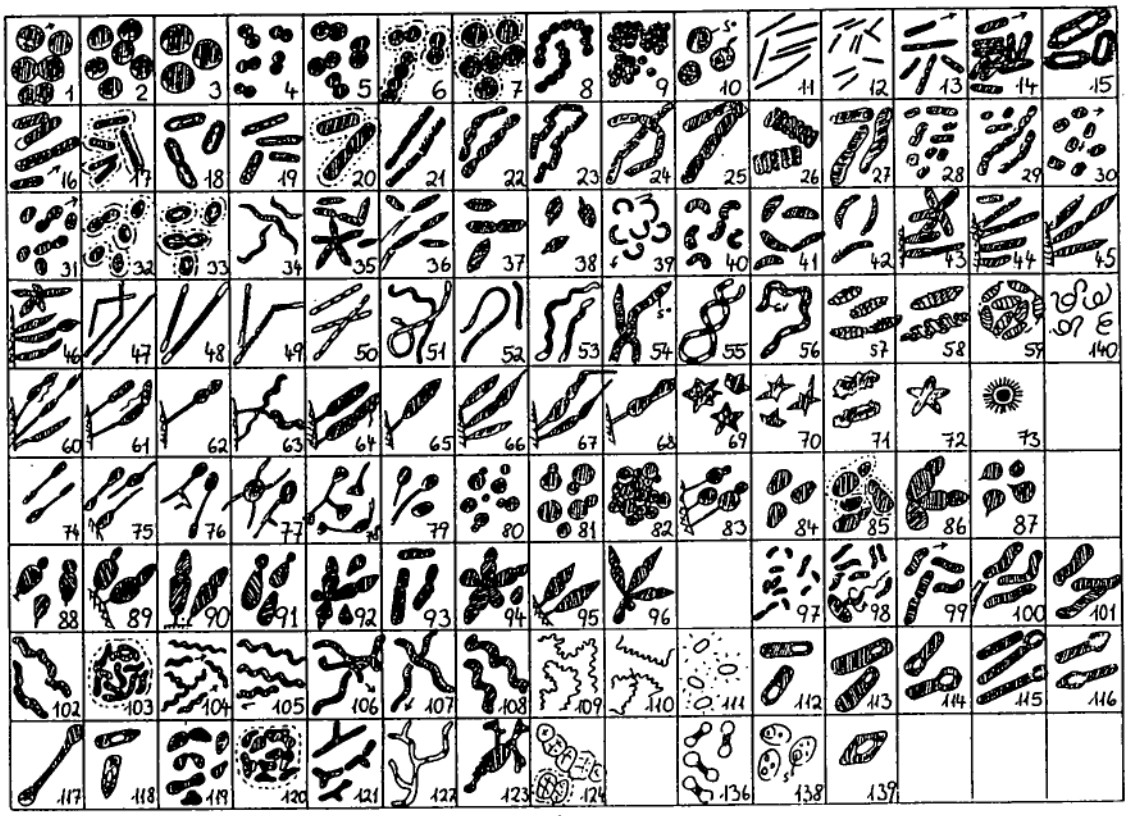


Figure 2.1: Visible evidence of microbial diversity in Ekho Lake. Morphotype table for prokaryotes from microscopy of biotin-surfaced glass slides incubated *in situ* (Hirsch & Siebert, 1991a).

input have also been recorded. "It is a common misconception that Antarctic aquatic ecosystems are very stable..." (Jones & Ellis-Evans, 1994). The Antarctic lake systems vary in both the short and long term. It is not surprising that the opportunistic biota which have colonised these environments are most often psychrotolerant and halotolerant. Psycrophiles will be the dominant organism only in stable cold environments (Baross & Morita, 1978), with obligate halophiles dominating stable hypersaline environments (del Moral *et al.*, 1987).

2.1.4 The Vestfold Hills Lakes: major reviews

The Vestfold Hills and its biologically, chemically and physically stratified lakes have been reviewed and examined by a large number of authors. The Vestfold Hills area is described in detail by Johnstone *et al.* (1973). Wright and Burton (1981) review early developments in the biology of Antarctic saline lakes. Pickard (1986) and Ferris *et al.* (1988) have written major reviews of the Vestfold Hills' climate, geomorphology, history, terrestrial and aquatic flora and fauna.

2.2 Growth parameters in cold saline water

Some microbial parameters are easily measured allowing generalisations to be derived. Those parameters that are more difficult to generalise may be modelled in simplified ecosystems if their components can be studied in detail (Pedrós-Alió & Guerrero, 1993). The Vestfold Hills offers a unique and localised opportunity to study the aquatic environment over a wide range of temperature and salinity. Reported conditions in the lakes range from -19 to 16 °C and from 0 to 28% salts (1.00 to 1.21 g ml^{-1}) (Ashbolt, 1990).

2.2.1 Species diversity in cold saline water

Adaptation to thermal and osmotic stress diverts energy from normal metabolic processes making a cell less able to compete. Basal metabolic processes take more energy to complete at reduced temperature and cells are less able to tolerate increased salinity (McMeekin *et al.*, 1993). The response of microbiota to large scale changes in environmental conditions is most likely to be by species replacement.

Species perturbations may not be so important from an ecological perspective if there is an overlap in niche and function. The ability of the microbial assemblage to react to change in environmental parameters is more important. A stressed community, or one lacking in diversity, may not be able to take full advantage of environmental change.

It has been suggested that the Antarctic environment may lack species diversity: "For many microbial species in the polar environment, niche partitioning may be dictated to a greater extent by their tolerance characteristics rather than by their competitive abilities. ...the corollary of this hypothesis, [is] that polar micro-organisms occupy broad 'fundamental' and 'realised' niches and that niche overlap is minimal." (Vincent & Quesada, 1994).

This is not only suggested of polar saline lakes. Williams (1991) noted that in the saline lakes of Greenland and southern Africa, lakes of relatively recent origin, the biota diversity is low. It is mostly comprised of that small fraction of the local freshwater biota which is tolerant to elevated salinity. Arntz and Gutt (1994), however, confront the tendency to draw general conclusions about diversity from very little evidence. They conclude that there seems to be no general pattern of species diversity over Antarctic marine environments or in taxonomic groups.

Whatever the case, the diversity of an aquatic environment may be modelled through the detailed study of species composition (see Section 2.3).

2.2.2 Metabolic activity and productivity

More than 80% of the Earth's biosphere is permanently cold (<5 °C). Microorganisms capable of growing at low temperature are therefore very important when considering global ecology (Russell, 1990). Microbial communities have been found at temperatures down to -10 °C and at salinities >15% in Southern Ocean pack ice (Garrison & Thompson, 1994). The lakes of the Vestfold Hills exhibit similar characteristics.

2.2.2.1 Metabolic cost of decreased temperature

Conventional literature indicates that the specific growth rate of a mixed bacterial consortia is positively correlated to temperature (Díaz-Ravina

et al., 1994; McMeekin *et al.*, 1991; Oren, 1990; Solic & Krstulovic, 1994). Enzymes adapted to lower temperature seem to be no less efficient than their mesophilic counterparts, apart from simple physical rate limitations described in the terms of the Arrhenius equation (Russell, 1990).

Below a certain temperature, reduction of metabolic activity must be countered by cryoprotective mechanisms. There is a large body of literature investigating cold-shock proteins, enzyme increase and modification of substrate uptake mechanisms, reorganisation of membrane lipids and the synthesis of short chain, branched chain or unsaturated fatty acids. This is summarised by Russell (1990) who concluded that ability to grow at low temperature must be an overall cellular phenomenon. Nichols *et al.* (1995) believe that in Antarctic psychrophylic bacteria the ability to manipulate cellular fatty acid composition is a key physiological adaptation. The energetic cost of growth at low temperature is compounded by a general reduction in substrate affinity at those temperatures. Nedwell & Rutter (1994) state that this decreased affinity is common to psychrophilic and mesophilic bacteria, data was not found for thermophilic bacteria but they postulate that this low temperature inhibition of uptake is common. The metabolic cost of growth at low temperature is reflected in the greater requirement for nutrients by bacteria grown at lower temperature (Wiebe *et al.*, 1992).

2.2.2.2 Metabolic cost of increased salinity

The stress from increased salinity is primarily osmotic stress (Galinski & Trüper, 1994). Decreased water activity (a_w) from increase in external solute concentration causes dehydration of most cells. Internal electrolyte concentration is increased by dehydration and eventually solutes precipitate causing changes in cytoplasmic composition and pH. High concentrations of electrolytes are toxic to most bacteria (Mazur, 1980). The activities of enzymes and metabolic processes within the cell are sensitive to changes in internal ionic concentration or concentration of solutes. Halotolerant bacteria take up external solutes or synthesis compatible solutes to avoid the harmful effects of low a_w. Accumulation by uptake is energetically preferable to biosynthesis (Imhoff, 1986), though accumulation by either method places energy demands on the cell (Csonka, 1989). Accumulation of compatible solutes is a useful

method of osmoadaptation as they may be readily excreted under dilution stress (Galinski & Trüper, 1994). The development of halotolerance can also involve changes to the lipid content of cells, synthesis of new proteins, changes in membrane fluidity or the ability to maintain membrane potential (Hamaide *et al.*, 1984). All of these processes are energetically expensive.

McMeekin *et al.* (1993b) extrapolated bacterial growth rate data for *Staphylococcus xylosus* over a range of a_w and determined that the theoretical minimum growth temperature (T_{min}) remained constant. They found that the actual minimum temperature at which growth occurred increased as a_w decreased. From this they were able to calculate the amount of the cells energy being diverted to counteract the effect of lowered water activity. In the case of *S. xylosus* approximately 200 kJ mol^{-1} was needed to overcome a decrease from an a_w of 0.98 (optimal growth a_w) to an a_w of 0.85.

Many halophilic bacteria have pumping mechanisms for excluding the Cl^- ions which inhibit intracellular processes (Kushner, 1988). In the eubacterial halophiles, protein synthesis and most intracellular enzymes, are inhibited by concentrations of NaCl at which the cells can grow. Due to the action of Na^+ pumps, intracellular free Na^+ can be lower than 20% of the extracellular medium and does not seem to vary with external salt concentration (Gilboa *et al.*, 1991).

McMeekin *et al.* (1987) characterised the growth rate of microorganisms in response to temperature and water activity (a_w). They concluded that the effects of temperature and a_w were additive. This hypothesis has been supported by other workers, "...these two factors act independently on microbial growth in the growth phase." (Davey, 1989). Generation of cryoprotectants (compounds present in the cytoplasm which are able to protect the cell from the deleterious effects of low temperature) is likely to divert as much energy from the cell as the generation of compatible solutes. Some cryoprotectants such as trehalose can also act as compatible solutes (Russell, 1990). Here the energy cost to the cell may be lower due to the possibility of dual action.

2.2.2.3 a_w effects of ice formation

"The lower growth limit [of cells] is fixed by the freezing properties of dilute aqueous solutions inside and outside the cell." (Russell, 1990).

Between 25 °C and 0 °C, a_w shows little variation with temperature (Chirife *et al.*, 1982). When salt water is cooled to its freezing point, water (solvent) crystallises out of solution as ice. This concentrates the salts (solute) in the remaining liquid (brine). Increase in salinity causes an increase in freezing point depression (FPD) and further freezing will only take place with a decrease in temperature. The FPD for a particular concentration of solutes depends upon the composition of those solutes. For sea water salts (SWS) from 0-6% the relationship is:

$FPD(seawater) = -3.1432x10^{-2} + 0.58004 \times \%SWS - 1.0445x10^{-2} \times \%SWS^2$ ($r^2 = 1.000$)
(Weast, 1973)

For a NaCl solution from 0-23% the relationship is:

$FPD(NaCl) = 0.22918 + 0.44545 \times \%NaCl + 1.8813x10^{-2} \times \%NaCl^2$ ($r^2 = 0.999$)
(Weast, 1973)

Below the freezing point a_w is dependant only on temperature, irrespective of the nature of the solute (Troller & Christian, 1978). FPD can be used to calculate the a_w of a brine and the temperature below zero (TBZ) will give the a_w of ice:

$-\ln a_w = 9.6934x10^{-3} \times (FPD \text{ or } TBZ) + 4.761x10^{-6} \times (FPD \text{ or } TBZ)^2$ ($r^2 = 1.000$)
(Ferro-Fontán & Chirife, 1981)

If the TBZ of the system is the same as the FPD of the solute concentration, the ice and brine phases are in equilibrium (i.e. equal vapour pressures) and the a_w is the same for both.

Once the temperature drops sufficiently, the solution becomes saturated and both solute and solvent precipitate out at an equal rate (the eutectic point) but the vapour pressure of the ice continues to decrease. For

seawater salts this point is below -20 °C and can be discounted for the purposes of this discussion.

The formation of ice in any limnological system affects the associated bacteria. Bacteria may become trapped in the ice matrix, be located totally within the brine exudate or be associated with both the ice and the brine. In such cases, the cell experiences a decrease in a_w determined solely by the TBZ, which must be compensated for in the same manner as the cell would for an increase in external solute concentration.

This freezing point effect is far more physiologically challenging than increase in external solute concentration. It is virtually instantaneous (with change in TBZ) whereas change in external solute concentration may occur over longer periods.

The minimum limit to growth appears to be -12 °C (Mazur, 1980) at which point the a_w experienced by the cell would be 0.89, equivalent to a solution containing 15% NaCl. However various species of bacteria, yeasts and moulds can grow at a_w below 0.89. Species from the genus *Halobacterium* can grow down to an a_w of 0.76, equivalent to the a_w effect of -29 °C. At such temperatures the ability to 'grow' may be a moot point; below -12 °C metabolic activity would not meet minimum cell maintenance requirements (Mazur, 1980).

The hypothetical effect of temperature and salinity on each aspect of cell physiology mentioned above can be modelled from a community perspective (Figure 2.2) using the ratio of metabolic activity to productivity. The axes used in Figure 2.2 are justified in Section 2.4.3. Minimum metabolic activity per unit of productivity is seen under optimal growth conditions (Figure 2.2, point A). The ratio between metabolic activity and productivity will increase due the unavoidable metabolic cost of increasing salinity (Figure 2.2, point B) and decreasing temperature (Figure 2.2, point C). These effects are additive (Figure 2.2, peak D). Below the freezing point, temperature effects are compounded by an additive a_w effect (Figure 2.2, peak E) due to the vapour pressure of ice at sub-zero temperature (Troller & Christian, 1978). This effects the cell in a similar manner to an increase in external salinity.

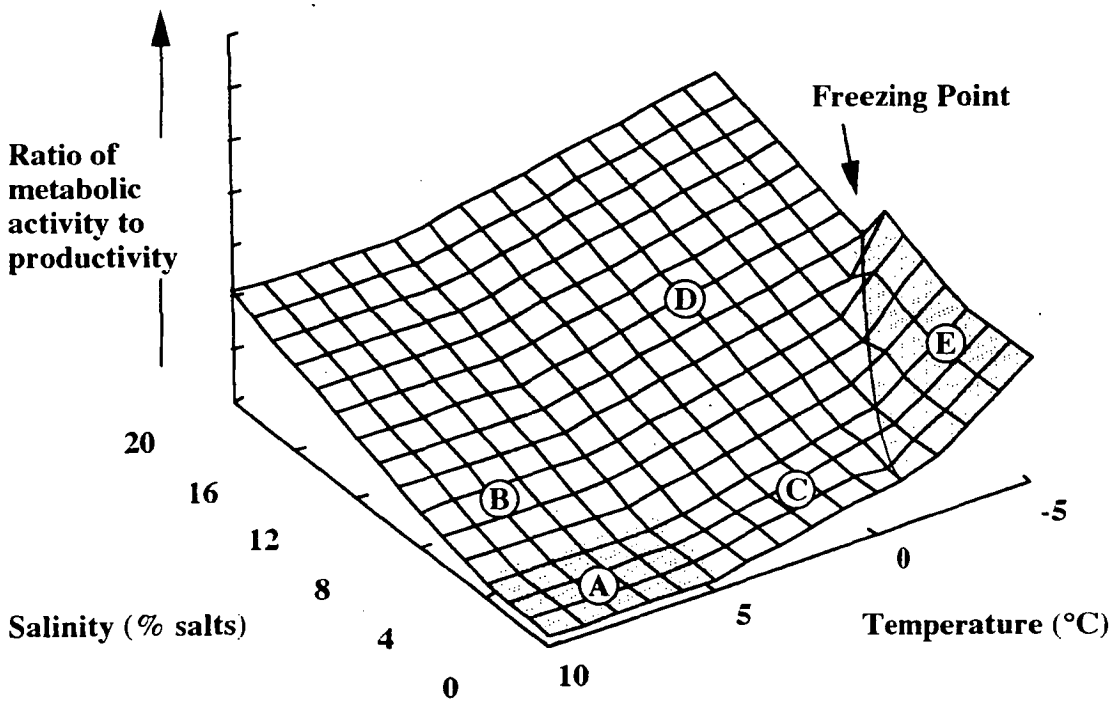


Figure 2.2: Surface plot of ratio of metabolic activity to productivity versus salinity and temperature. Minimum ratio of metabolic activity to productivity is seen under optimum growth conditions (point A), the ratio will increase with increasing salinity (point B) and temperature (point C). These effects are additive (point D). Below freezing point, temperature effects are compounded by an additive a_W effect (point E).

2.3 Direct enumeration of microbiota by immunofluorescence microscopy

A bacterial species which is morphologically unique and identifiable through its lifecycle can be quantified within the natural aquatic environment by normal microscopy. Most culturable bacteria found in Antarctic sea water (Delille, 1993) and continental lakes are Gram-negative rods which are unidentifiable solely by morphology. These need to be quantified by some other method.

The technique of linking fluorochromes to antibodies was developed more than 60 years ago (Chantler & McIllmurray, 1987). Identification of bacterial species by immunofluorescence was first used in 1965 by Unger & Wagner (Hill & Gray, 1967) who used fluorescent antibodies to identify colonies of soil bacteria on dilution plates. Danielsson & Laurell investigated the detection of bacteria in water in 1965 (Dahle & Laake, 1982). During the 1970s and late 1960s this technique was substantially developed, primarily as a method of visualising soil microorganisms. A number of early papers dealing with this technique are reviewed by Dahle & Laake (1982).

Antibacterial polyclonal antibodies are often very strain specific, others are specific to the species used as an antigen. There is very little cross-reactivity with other bacterial species (Dahle & Laake, 1982; Wilberg *et al.*, 1993). This technique can provide specific information on the diversity of a microbial community and the proportion of non-culturable to culturable bacteria within that community. Unlike polyclonal antibodies, monoclonal antibodies are usually consistent across batches. Also, they are more specific. However the generation of monoclonals is more difficult and used less often for the monitoring of general environmental bacteria (Chantler & McIllmurray, 1987).

2.3.1 Indirect immunofluorescence method

In the indirect immunofluorescence method the primary reaction between the antibacterial antibody and the bacterial antigen is visualised using antibodies (labelled with a fluorochrome such as fluoroscein isothiocyanate (FITC)) which bind to the antibacterial antibody. The labelling of a secondary antibody avoids deleterious effects on the

biological activity of the antibacterial (primary) antibody by the labelling process (Chantler & McIllmurray, 1987).

Alternative methods include direct immunofluorescence or measurement of the degree of clumping by agglutination reactions. Indirect staining gives a clearer result with 5-10 times the sensitivity of these alternative methods (Kawamura, 1969) as several fluorescently labelled antibodies may bind to each antibacterial antibody (Figure 2.3). This brighter fluorescence is important for differentiating specific fluorescence from unwanted fluorescence (cross-reactivity) and auto-fluorescence.

Fluorescence intensity decreases after exposure to light (fading) and can be influenced by pH. Alternative labels such as radioisotopes, enzymes and electron-dense particles do not have these particular problems but are more difficult to quantify (Chantler & McIllmurray, 1987).

Methods of raising antisera deal with different types of animals and various species of bacteria (or other antigens), so potency and specificity must be optimised on a situation by situation basis. Some aspects are common to most methodologies; purifying the somatic antigen, immunoresponse feedback to optimise immunisation schedule, isolation of most potent antibody fraction from the serum, comprehensive evaluation of the antibodies and use of appropriate control materials (Chantler & McIllmurray, 1987; Kawamura, 1969). An adjuvant increases the immune response when co-administered with the antigen, though the precise reason for this is unclear (Lillehoj & Malik, 1993). Freund's adjuvant is a common choice, it is a mineral oil/water emulsion containing suspended mycobacteria.

For increased specificity, antisera can be absorbed onto preparations of the antigens which exhibit the unwanted reaction (cross-reactivity). This usually decreases the potency of the remaining antisera. It is common for antisera to show some general background reaction to many microorganisms (natural antibodies).

Environmental scientists tend to use polyclonal antibodies as they are comparatively simple to produce. Many recent developments in antibody technologies which increase antibody yield and specificity are covered in a review by Lillehoj & Malik (1993).

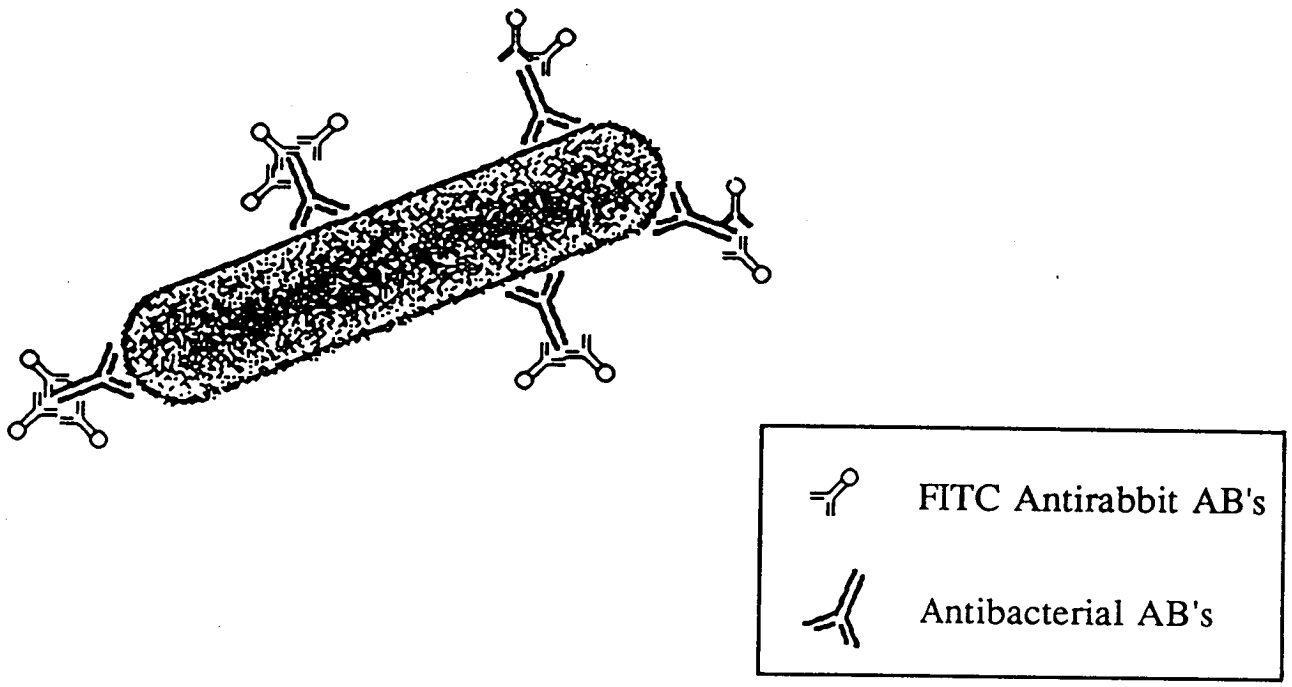


Figure 2.3: Diagram showing FITC conjugated antirabbit antibodies bound to rabbit-raised antibacterial antibodies bound to the antigenic bacteria. Antibody is abbreviated to AB.

2.3.2 Enumeration by immunofluorescence versus other methods

Early immunofluorescence studies on the identification of bacteria within natural marine populations include; Dahle & Laake (1982), Reed & Dugan (1978), Ward & Perry (1980) and Xu *et al.*, (1984). Recently the technique has lost popularity due to the specificity and promise shown by methods of DNA hybridisation probing.

It is commonly accepted that culturing on selective media is a poor way of measuring microbial diversity. Wayne *et al.* (1987) state that less than 20% of *in situ* bacteria are isolated using this method, less than 1% is more probable (Cheetham, 1987). Delille *et al.* (1988) investigated marine samples by frequency of dividing cells (FDC) and found some active cells were present *in situ* which did not grow on the culture media tested. The capacity of bacteria to grow on media clearly decreased with depth. Many bacteria are difficult or impossible to culture under laboratory conditions (Fani *et al.*, 1993) and even the most basic molecular techniques reveal that most inhabitants of the natural microbial community have never been cultured (Torsvik *et al.*, 1990; Ward *et al.*, 1990). With sufficient culturable species, antibodies may be generated to actively monitor species diversity within an ecosystem but this situation is unlikely to occur as a number of recent molecular techniques can monitor diversity far more easily. These techniques include; DNA hybridisation probing (Giovannoni *et al.*, 1988; with flow cytometry, Amann *et al.*, 1990), cloning (Bond *et al.*, 1995; Fuhrman *et al.*, 1993) or denaturing gradient gel electrophoresis (Muyzer *et al.*, 1993) followed by restriction fragment length polymorphism (Moyer *et al.*, 1994), partial sequencing or DNA hybridisation probing of the partial sequences, or random amplified polymorphic DNA techniques (Fani *et al.*, 1993). Nevertheless the immunofluorescent antibody techniques are an accurate and accessible method of obtaining an understanding of the culturable species within a system.

White (1994) suggested that analysis of signature lipid biomarkers answers more questions about community structure and activity than nucleic acid analysis. Lipid biomarkers can, to varying degrees, indicate; viable biomass, quantitative community structure, nutritional status, toxicity, unbalanced growth or physiological stress, Gram reaction, lysed cells and terminal electron acceptor (respiratory quinone structure). Additionally, signature lipid biomarker analysis and nucleic acid

analysis (for specific gene frequencies) are compatible, potentially giving a more comprehensive description of community structure. An earlier review of lipid biomarkers is given in Tunlid and White (1990).

Flow cytometry also offers markedly improved precision and accuracy over traditional epifluorescence microscopy (Karl, 1994). The principles of flow cytometry including; cell sorting, measurement of cell subsets using antibodies, DNA-RNA measurement and estimating cell viability are covered in Ormerod (1990). Amann *et al.* (1990) investigated probing with oligonucleotides in mixed microbial communities using flow cytometry. In the absence of a sorting flow cytometer, immuno-magnetic beads coated with polyclonal antibodies have been used to capture particular bacteria from environmental samples (Christensen *et al.*, 1992; Morgan *et al.*, 1991).

Though staining with fluorescently labelled polyclonal antibodies permits specific counts of bacteria, the technique does not differentiate active from inactive cells (Hicks *et al.*, 1992). However, radioisotope uptake will give an indication of bacterial activity, at least on a community level.

2.4 Radioisotope incorporation

2.4.1 [*methyl*-^{3}H]thymidine incorporation

Roodyn and Mandel first measured bacterial production by [*methyl*-^{3}H]thymidine (Tdr) incorporation in 1960 (Pollard & Kogure, 1993). Brock (1967) analysed bacterial growth rates using Tdr incorporation on a monoculture in marine water. Thomas *et al.* (1974) measured the activity of soil organisms using Tdr incorporation but used long incubation times, probably labelling both bacterial and eukaryotic DNA (Christensen *et al.*, 1989). Moriarty and Pollard (1981) and Fuhrman and Azam (1980; 1982) brought the technique into popular use.

The incorporation of Tdr as a method of measuring bacterial productivity has been the subject of criticism, "...measuring the metabolic activity of a community is not technically difficult; the problem is how to interpret the results." (Staley & Konopka, 1985). Nevertheless the technique is widely used and remains a valuable tool for measuring whole

community activity (Moriarty, 1986; Staley & Konopka, 1985; Robarts & Zohary, 1993).

Cell division and the synthesis of DNA are directly correlated (Moriarty, 1986). Incorporation of sufficient exogenous Tdr limits *de novo* synthesis of deoxythymidine monophosphate by feedback inhibition (Figure 2.4). Tdr incorporation offers an important advantage over the incorporation of other radiolabelled DNA bases as it is not incorporated into RNA. The production of RNA responds dramatically to growth rate (Staley & Konopka, 1985). Tdr taken up under the salvage pathway is used primarily for DNA synthesis so is closely associated with productivity.

Key assumptions in the method include: (i) that only bacteria incorporate Tdr; (ii) all actively dividing bacteria incorporate Tdr; (iii) all cold trichloroacetic acid (TCA) precipitable Tdr is incorporated into DNA; and (iv) extracellular dilution can be accounted for (Figure 2.5) (Davis, 1989).

2.4.1.1 Specificity of [methyl-^{3}H]thymidine incorporation

Tdr uptake is mediated by a specific enzyme, thymidine kinase, which is not present in most algae and fungi. Pollard & Moriarty (1984) showed that there was no significant incorporation of Tdr by three species of microalgae. *Tetraselmis* sp., common to the hypersaline lakes of the Vestfold Hills (Hirsch & Siebert, 1991a), does not incorporate Tdr into DNA (Martinez *et al.*, 1989). In a study by Wellsbury *et al.* (1993), actively growing cultures of sulphate-reducing bacteria, purple sulphur bacteria, methanogens and acetogens did not incorporate Tdr. Of the cultures tested (n=24), only a facultatively anaerobic *Bacillus* sp. and members of the genus *Clostridium* incorporated Tdr into DNA. Riemann and Bell (1990) noted that Tdr was not taken up by chemolithotrophs or sulphate reducers. Anaerobic bacteria may lack thymidine kinase and so cannot incorporate Tdr (Moriarty, 1986; Winding, 1992). Only very few members of the anaerobic community will incorporate Tdr, therefore this technique will underestimate production in the anaerobic environment.

Not all heterotrophic bacteria can take up Tdr. Mitchel and Bloem (1993) found that six out of ten soil isolates incorporated Tdr, while all

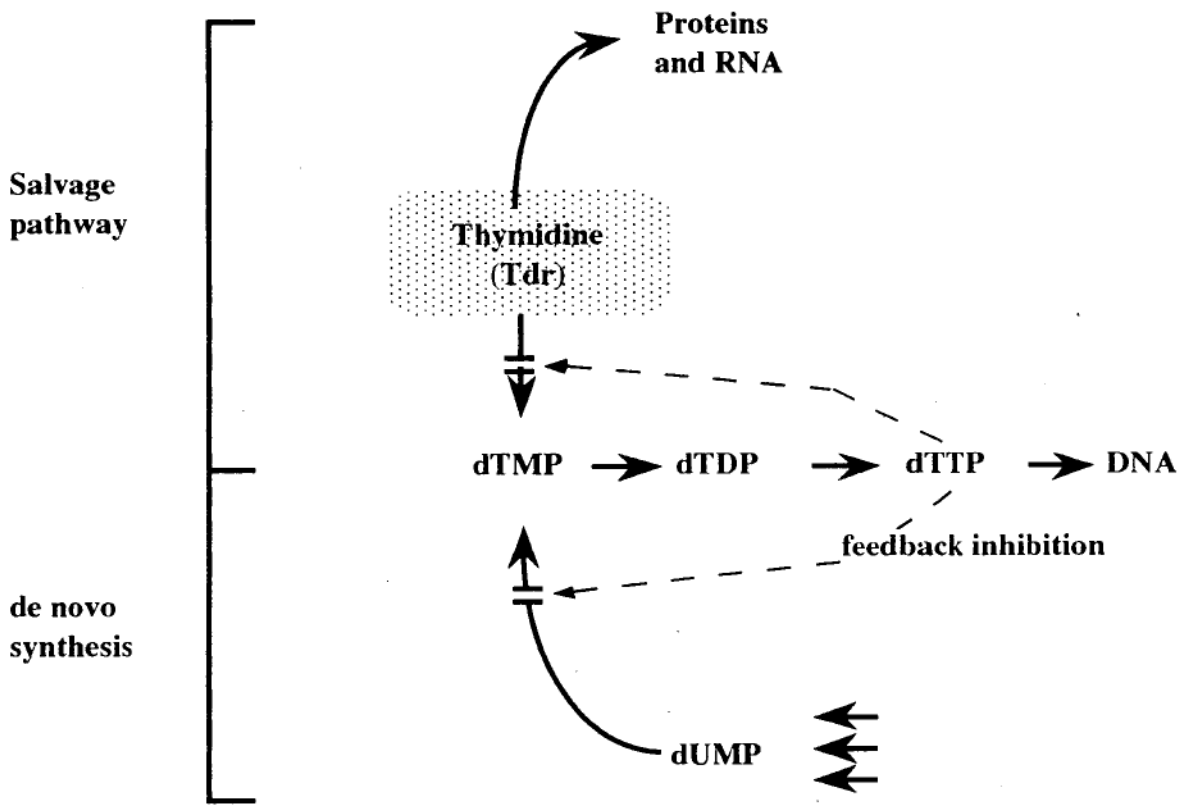


Figure 2.4: Pathways of Tdr metabolism with feedback inhibition. Thymidine is converted successively to thymidine mono-, di- and tri-phosphate before being incorporated into the DNA. From Moriarty (1990).

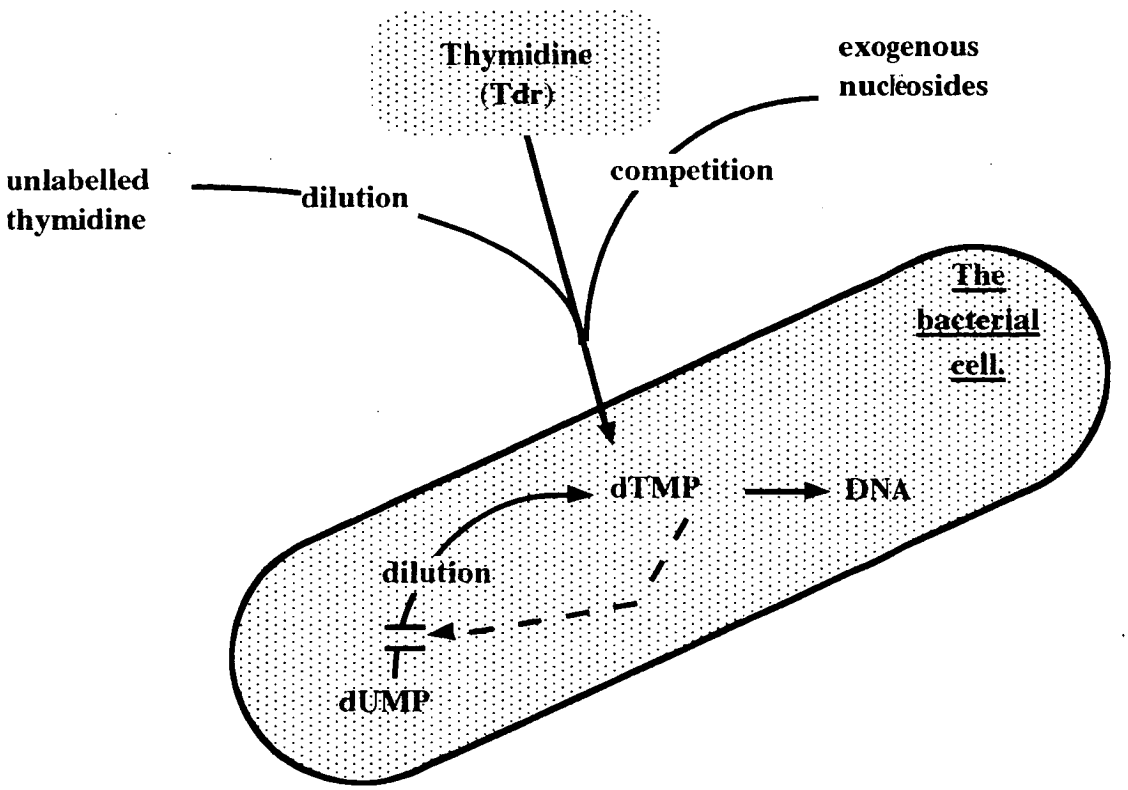


Figure 2.5: Possible sources of isotope dilution during the uptake and incorporation of Tdr.

incorporated L-[3,4,5-^{3}H]leucine (Leu). Of 34 strains isolated from seawater by Davis (1989), four were unable to incorporate Tdr. These were identified as belonging to the genera *Vibrio*, *Pseudomonas* and *Flavobacterium*. Strains of other genera; *Vibrio*, *Cytophaga*, *Serratia*, *Pseudomonas* and *Flavobacterium*, were able to take up and incorporate Tdr. Two isolates (a *Pseudomonas* sp. and a *Cytophaga* sp.) were able to incorporate Tdr presented at low concentrations (9.1 nM) but were unable to incorporate Tdr at higher concentrations (19 nM). Uptake rates varied considerably from strain to strain and were substantially lower in starved cells. Pollard and Moriarty (1984) showed that two species of *Pseudomonas* were unable to incorporate Tdr but that some strains of the genera *Alcaligenes*, *Alteromonas* and *Pseudomonas* were able to incorporate Tdr.

Different strains of bacteria and similar bacteria in different physiological states have varying radioisotope uptake and incorporation rates (Davis, 1989). Uptake rate may exceed or be dependant on incorporation rate (Robarts *et al.*, 1986), so change in strain composition of the population or in physiological condition may influence incorporation rates. Any of these effects may reasonably be expected in the Antarctic lake ecosystems under study.

2.4.1.2 Alternatives to [methyl-^{3}H]thymidine incorporation

The most direct method of determining bacterial productivity would be to count FDC visually. Quantification of the FDC may be difficult as division of small cells may not be resolvable. Measurement of FDC is time consuming and difficult to calibrate satisfactorily (Azam *et al.*, 1983). In the words of Riemann and Bell (1990), "When image analysis is used more generally the FDC procedure will probably experience a renaissance in microbial ecology." Another technique for the measurement of *in situ* bacterial productivity is nalidixic acid cell enlargement (Kogure *et al.*, 1987). As in most environments, the cells normally found in the hypersaline lakes of the Vestfold Hills are extremely variable in length. Because of this the nalidixic acid method would be inappropriate.

Phospholipids are the major lipids in bacteria. Though phosphate is used in a variety of syntheses within the cell, during reproduction and growth phophate is mainly incorporated into phospholipids, these lipids are

rapidly turned-over within the cell and the rate of incorporation can be correlated with bacterial productivity. $H_3{}^{32}PO_4$ incorporation methods are similar those used for the incorporation of Tdr and are described in detail in Moriarty (1990).

ATP levels within a microbial community may also be used to quantify productivity though there are problems with its extraction and analysis and this technique is not so often used (White *et al.*, 1979).

2.4.1.3 [methyl-^{3}H]thymidine incorporation; assumptions

The underlying assumption that the technique results in Tdr being mostly incorporated into DNA is questioned by Robarts *et al.* (1986) who found that in some cases a significant portion of Tdr was incorporated into protein. The percentage incorporation of Tdr into DNA can be subject to diurnal, seasonal, spatial and methodological variations (Robarts & Zohary, 1993; Robarts *et al.*, 1986). This effect was attributed to periods of unbalanced growth. Specific criticisms of the technique are levelled by Karl (1994): (i) nonspecific catabolic labelling of macromolecules is recognised as ubiquitous; (ii) variable per capita uptake rates; (iii) uptake by starved non-growing cells; and (iv) large variation in variable, habitat-dependant conversion factors.

Virtually all aspects of the technique of radioisotope incorporation have been debated over the last ten years. There are many studies on Tdr incorporation techniques. In major reviews, Staley and Konopka (1985) and Moriarty (1986) cover literature on Tdr incorporation to 1985, presenting lists of rates of incorporation in various environments and summarising arguments about the technique. Albright and McCrae (1987) list a number of aquatic bacterial production rates. Jonas *et al.* (1988) list Tdr incorporation in various aquatic systems. Brittain and Karl (1990) review and investigate nonspecific labelling. Riemann and Bell (1990) cover aspects of the use of nucleic acid precursors.

The recent review of Robarts and Zohary (1992) collates the most pressing arguments and evidence about the technique of Tdr incorporation. This review synthesises information which has been published since Moriarty (1986).

Much of the contention over Tdr incorporation relates to application of conversion factors used to determine rates of bacterial division, bacterial biomass and productivity in terms of carbon. Tdr incorporation only measures DNA synthesis and gives an indication of increase in bacterial biomass only under conditions of balanced, regular growth. Cells increase their volume before they divide, only occasionally will cells divide without increase (Kogure *et al.*, 1987). At low temperature biovolume and generation times are markedly affected by substrate concentration. Wiebe *et al.* (1992) noted the effect on growth rate was particularly apparent at and below 5 °C for large increases in simple substrate concentration. The use of a constant cell volume in estimation of carbon production will produce systematic errors. A method of directly measuring protein synthesis, such as Leu incorporation, would provide a better indication of general cell activity.

2.4.2 [^{3}H]leucine incorporation

Kirchman *et al.* (1985) first suggested the use of Leu incorporation into protein to quantify bacterial metabolic activity. Incorporation of Leu as a measure of bacterial metabolic activity is based on the assumption that leucine is used by the bacteria and represents, or is equivalent to, the main available nutrient in the natural environment (Bölter, 1993). Like Tdr, Leu must be introduced in sufficient amounts to suppress de-novo synthesis without stimulating growth rate. Low concentrations are specific for heterotrophs, with their highly efficient uptake mechanisms (Kirchman *et al.*, 1985). Key assumptions in the method include: (i) that only bacteria incorporate Leu; (ii) all metabolically active bacteria incorporate Leu; and (iii) Leu is use primarily for protein synthesis. A small proportion of uptake in some bacterial strains may go to the synthesis of iso-branched odd-chain fatty acids (i13:0/i15:0) (Nichols & Russell, 1995).

van Looji and Riemann (1993) state that it is difficult to saturate the eutrophic system as, when using large concentrations of Leu, changes in bacterial protein synthesis might occur or the Leu may be utilised by other organisms. Maximal (saturated) incorporation rate can be extrapolated by Michaelis-Menten kinetics from plots of Leu incorporation rate against concentration of added Leu. It is not necessary to extrapolate maximum velocity of Tdr incorporation as the system becomes saturated with Tdr at attainable concentrations. Logan and

Feury (1993) recommended Wright-Hobbie plots as an alternative to Michaelis-Menten kinetics. This aspect of the calibration of Leu incorporation is yet to be resolved.

Most heterotrophic bacteria can take up Leu. Mitchel and Bloem (1993) found that six out of ten soil isolates incorporated Tdr, while all incorporated Leu. More than 90% of radiolabelled leucine, tyrosine, lysine and alanine is incorporated into protein. Incorporation of tritiated valine has also been used to estimate bacterial production (Servais, 1995). Growth rate studies indicate that leucine stimulates bacterial growth to a lesser extent than glucose (Bölter, 1993).

2.4.2.1 Alternatives to [^{3}H]leucine incorporation

Fluorescence microscopy, to estimate bacterial numbers, combined with electron microscopy, for estimating bacterial volumes, can provide an accurate estimate of bacterial biomass (Azam *et al.*, 1983). Other possible methods of measuring bacterial growth rates include the reduction of tetrazolium dye (but the intracellular granules reduced from tetrazolium may not be visible in small cells making it difficult to use in marginal environments (Staley & Konopka, 1985)), the measurement of respiring cells (Kogure *et al.*, 1987), estimation from the phospholipid-phosphate content of a sample (White *et al.*, 1979) or extraction of compounds unique to a group of organisms (Vestal & White, 1989). See also Section 2.4.1.2 for other methods that may be used to determine biomass.

Leu incorporation has become the most popular method of estimating bacterial protein synthesis in the environment and, with suitable conversion factors, rate of bacterial biomass increase.

2.4.3 Ratio of [^{3}H]leucine incorporation to [*methyl*-^{3}H]thymidine incorporation

Measurement of both Leu and Tdr incorporation is a valuable technique for providing information on the metabolic activity of an entire population. Under optimum conditions the generation of one bacterium requires approximately 10 times the incorporation of Leu into protein as Tdr into DNA (ratio of Leu:Tdr) (Riemann & Bell, 1990; Simon & Azam, 1989). It has been indicated that bacterial production and amino

acid metabolism are tightly coupled in the marine system (Jonas *et al.*, 1988; Servais & Garnier, 1993). Field observations generally produce good correlations between the incorporation of Tdr and Leu (van Looji & Riemann, 1993). Average ratios in marine or lake systems mentioned by other authors: 5 (Grossman & Dieckmann, 1994); 7-11 (Kirchman, 1990); 2-26 (Riemann & Bell, 1990); 6-9 (Servais & Garnier, 1993) are similar to this optimum value. Not all environments exhibit the same molar ratios. In soil, measured values are higher: 35-51 (Bååth, 1994); 14-31 (Díaz-Ravina *et al.*, 1994); 51-113 (Tibbles *et al.*, 1992). In young pack ice the ratio value was 1 (Grossman & Dieckmann, 1994).

Though the incorporation of Leu and Tdr are correlated under normal conditions, this is not the case for cells under stress. Protein turnover rate increases under slow growth or starvation conditions (Snyder *et al.*, 1994). There is a strong correlation between decreased growth efficiency and slower growth rates. As generation time increases, more Leu is incorporated per unit of bacterial production (Snyder *et al.*, 1994). In cold or saline environments where generation time is measured in days, this metabolic cost becomes very significant.

Slezak *et al.* (1994) found that the incorporation of Tdr and Leu decreased with decreasing pH (caused by acrylic acid) in marine samples, but the ratio of Leu:Tdr remained constant. The ratio of Leu:Tdr also remains constant with increasing pressure. Pressure disadvantages the production of protein and DNA equally (Turley, 1993).

Bacteria at lower temperature demonstrate an increased requirement for nutrients (Wiebe *et al.*, 1992), this would also act to increase the ratio of Leu:Tdr. The higher demand for amino acids for protein synthesis, compared with the demand for thymidine for DNA synthesis, would mean that the ratio of Leu:Tdr would increase with decreasing temperature.

Molar ratio has also been shown to increase with increasing cell size. Protein content increases with cell size but DNA is usually constant per cell (Servias & Garnier, 1993). Delille (1993) found that average cell volume increased during winter in his Antarctic ocean studies and noted that an inverse relationship between cell volume and temperature had been reported previously. Larger cells require proportionally more

protein synthesis than DNA synthesis and would require more maintenance energy. Wiebe *et al.* (1992) also found that aquatic bacteria increased cell size with lower temperature and reported that this was related to a decrease in generation time. Both effects would lead to an increase in the ratio of Leu:Tdr at low temperature.

Substantial decrease in the ratio of Leu:Tdr at low temperature such as in young pack ice (Grossman & Dieckmann, 1994) and in soils (Díaz-Ravina *et al.*, 1994) may indicate cell division by non-growing cells, i.e. cell division without commensurate increase in protein synthesis. This strategy is often used by nutrient limited cells to increase cell surface-area (Nystroem & Kjelleberg, 1989). The small size of bacteria limits the ratio of surface area to volume and assists in substrate uptake. Smaller cells will have the same amount of DNA but less protein, maintaining the decreased molar ratio (Servais & Garnier, 1993).

Shiah and Ducklow (1994) demonstrate that Leu and Tdr incorporation decrease at about the same rate to 5 °C. Looking at Leu incorporation only, Tulonen (1993) found a biphasic relationship between temperature and bacterial productivity, productivity being approximately linear above 5 °C and decreasing linearly with temperature below 5 °C. Wikner and Hagström (1991), measuring Tdr incorporation, found a similar relationship below 6 °C. Data from a large set of experiments showed that heterotrophs from sea ice microbial communities had maximum growth from around 4 to 15 °C; bacterial activity substantially decreased below 4 °C (Kottmeier & Sullivan, 1988). Pomeroy & Wiebe (1988) found that at temperatures above 4 °C heterotrophic bacteria utilise available substrates quickly; closer to 0 °C bacterial activity is suppressed by an unknown mechanism. On the basis of these studies the ratio of metabolic activity to productivity (indicated in these studies by Leu and Tdr incorporation) is shown in the hypothetical model proposed in Section 2.2.2.3 to increase below 5 °C (Figure 2.2, point C).

There are relatively few papers on the effects of hypersalinity. Oren (1990) found no correlation between salinity increase and productivity in solar salterns. Kottmeier & Sullivan (1988) found that for sea ice microbial communities' rates of carbon fixation, incorporation of Tdr and incorporation of uridine were maximal at about 3% salinity and declined markedly between 5 - 7% salinity. Most marine bacteria can grow in media with up to 5% salts (density $\approx$1.04 g ml^{-1}) but few can

grow in media with greater than 5% salts. From these trends the ratio of metabolic activity to productivity is shown in the hypothetical model proposed in Section 2.2.2.3 to increase above 5% salinity (Figure 2.2, point B).

2.5 Significance of the dissolved organic carbon pool

The dissolved organic carbon (DOC) pool comes from a variety of sources including runoff, excretion, phytoplankton exudate and autolysis. Grazing ciliates release low to intermediate molecular weight compounds which are rapidly incorporated and respired by heterotrophic bacteria (Taylor *et al.*, 1985). Much of the DOC pool is thought to be refractory but, by virtue of their abundance, high substrate affinities and rapid growth rates, bacteria are able to utilise much of the dissolved carbon. This is the labile fraction (Chrzanowski & Hubbard, 1988).

Organic matter which can pass through a 0.5μm - 1.0μm filter is conventionally described as 'dissolved', the coarser fraction being referred to as 'particulate' (Ogura, 1977). The possibility that filtration may increase DOC through rupture of fragile cells has been reviewed in Nagata & Kirchman (1990).

DOC release associated with algal death is dominated by relatively simple organic material such as amino acids, carbohydrates, organic acids and nucleotides (Bratbak, 1987). This is rapidly taken up by bacteria, creating particulate organic carbon which can be utilised by bacteriovorous protozoa. Bacteriovory releases carbon previously fixed in the bacterial biomass, stimulating bacterial activity. Bacteria may promote algal death by breaking down extra-cellular organic material essential for algal survival. The net effect of this microbial loop is to recycle nutrients making a second algal bloom possible (Bratbak, 1987). "It appears the control of carbon flow into bacteria depends on interactions between phytoplankton, bacterium, viruses and protozoa." (Azam *et al.*, 1993).

The role of bacteria in carbon fluxes within the marine environment is under review. Current ideas were synthesised by Azam *et al.* (1993) who emphasise recent discoveries: (i) bacterially mediated carbon flux is highly variable (0 to >100% of local primary production); (ii) depending on nutrient status, bacteria can switch between being net-consumers and

net-producers; (iii) new types of particles have been found, confusing the definitions of particle-bound and free-living; (iv) dissolved polymers play a pivotal role in bacteria-organic matter coupling; (v) pelagic bacteria are capable of bursts of very rapid growth, seemingly without a lag phase; (vi) utilisable organic matter may quickly become unavailable, perhaps due to condensation with carbohydrates; (vii) bacterial abundance generally remains within narrow limits even during plankton blooms, presumably due to predation by protozoa and viruses; and (viii) bacteria can become competitors of phytoplankton for nitrogen and phosphorous.

The controversial question of whether bacteria are a link (transferring energy to higher trophic levels) or a sink is as important in lake systems as it has become in marine systems (Acosta Pomar *et al.*, 1993). Alongi (1994) working in tropical mangrove systems, indicated that bacteria are important mineralisers of organic detritus and recyclers of essential nutrients, roles more important to the ecosystem than their trophic function. The role of bacteria may be more than just food for protists and invertebrates. As the primary decomposers of organic matter they act as a sink for carbon, processing most of the nutrients in tropical aquatic systems.

A new method of measuring DOC, high-temperature catalytic conversion, has produced some controversial estimates of the oceanic DOC pool and the rate of DOC turnover mediated by bacterioplankton (Kirchman *et al.*, 1991; Toggweiler, 1992). In their contribution to the debate on the new high-temperature catalytic oxidisation methods, Ogawa & Ogura (1992) suggest that the highly labile low molecular weight fraction of the DOC is underestimated by the wet chemical oxidisation technique by as much as 30%.

The oceanic DOC pool turns over very slowly and the question remains, as to why no bacterium or bacterial consortia has been able to exploit it (Azam *et al.*, 1993). The lakes of the Vestfold Hills can have very high concentrations of unexploited DOC, Organic Lake being an example of such.

While there is a large body of data on DOC quality and degradation in marine waters, there is a paucity of data on hypersaline waters. Investigating the ecological significance of the low-temperature polar

regions Nedwell and Rutter (1994) indicated that low temperature reduces substrate uptake. This may be significant in explaining the limitation of primary production in the Southern Ocean. They hypothesise that, "...in different areas of polar oceans primary production may be limited by different nutrients or combinations of nutrients... further complicated by the decrease in affinities for substrates at low temperature."

In aquatic systems newly introduced labile DOC can become refractory in a matter of hours most often due to adsorption onto organic floccules (Keil & Kirchman, 1993). If bacterial uptake of DOC is slowed by environmental factors, then newly introduced DOC may have time to become refractory. At a lower rate, refractory DOC may become useable through the action of photochemical degradation by UV radiation or other factors (Azam *et al.*, 1993; Mopper *et al.*, 1991).

3. MATERIALS AND METHODS

3.1 Preparation of antibacterial antibodies

The following protocol for antibacterial antisera production was developed from the methods of Kawamura and Aoyama (1983) and Gerencser (1979).

Bacterial cells were harvested from half strength artificial Organic Lake peptone yeast (1/2AOLPY) agar plates into 1/2AOLPY broth (Appendix 1.2.1). Cells were collected by centrifugation and the cell pellet weighed, resuspended in 1/2AOLPY broth with 6% formaldehyde (w/v) and incubated for 1 hr at room temperature. Cells were collected and resuspended in 1/2AOLPY broth to a density of approximately $1x10^{10}$ cells ml^{-1}. Formaldehyde was added to a final concentration of 0.25% (w/v). Ten New Zealand white rabbits were immunised with these cell suspensions during a 50 day period. Suspensions were combined either in equal proportions with Freund's complete adjuvant for intramuscular and subcutaneous injections or used alone for intravenous injections.

A pre-immunisation bleed of 10 ml on day one was followed by immunisation with a 2 ml subcutaneous injection of the cell suspension and a 3 ml intramuscular injection of the cell suspension. On day 28, the rabbits received a 0.5 ml intravenous injection of the cell suspension. On day 35, the rabbits received a 1 ml intravenous injection of the cell suspension. Finally, on day 42, the rabbits were bled from the marginal ear vein.

Serum antibody titre was checked by slide agglutination. One drop of the appropriate cell suspension was added to one drop of either normal (pre-immunisation bleed) serum or trial bleed serum diluted with 3% NaCl (w/v) to 1/10, 1/100, 1/1000 and 1/10000 concentrations. Cell/serum combinations showing agglutination after incubation for 1 hr at 37 °C at serum dilutions of 1/1000 or greater were considered acceptable.

Rabbits showing acceptable titres were bled at four day intervals. In most cases 30 ml of serum was obtained. Other rabbits were intravenously injected with 1 ml of the cell suspension on days 44 and 46. Then the trial bleed was repeated on day 50.

Serum globulins were precipitated with anhydrous sodium sulfate and purified using a Biogel P-6DG column. Sodium azide, at a final concentration of 0.01% (w/v), was added to all purified antisera as a preservative. Antisera were frozen using liquid nitrogen then stored at -20°C.

The four bacterial strains used to raise antibodies were obtained from the Australian Collection of Antarctic Microorganisms (ACAM). Slide agglutination was used to determine specificity of these antisera against different strains of the test species and other species within the family *Halomonadaceae*. Indirect immunofluorescence was used to confirm these results. No other species of the genus *Flavobacterium* were tested for antisera reactivity as *Flavobacterium gondwanense* and *F. salegens* are the first halophilic species of the genus *Flavobacterium* to be described (Dobson *et al.*, 1991).

Bacterial strains used in cross reactivity testing were; *Deleya halophila* (CCM 3662), *D. aesta* (NCMB 1980), *D. aquamarina* (DSM 30161), *D. pacifica* (DSM 4742), *D. cupida* (DSM 4740), *D. venusta* (DSM 4743), *D. marina* (DSM 4741), *Halomonas halmophila* (NCMB 1971), *H. elongata* (ATCC 33173), *Shewanella putrefaciens*. (ACAM 341) and an environmental *Pseudomonas* sp. (ACAM 162). A number of different strains of the four test species were also examined including; *H. meridiana* (ACAM 246T), *H. meridiana* (ACAM 233), *H. meridiana* (ACAM 235), *H. subglaciescola* (ACAM 12T), *H. subglaciescola* (ACAM 227), *H. subglaciescola* (ACAM 222), *H. subglaciescola* (ACAM 251), *H. subglaciescola* (ACAM 243), *H. subglaciescola* (ACAM 230), *H. subglaciescola* (ACAM 255), *H. subglaciescola* (ACAM 247), *F. gondwanense* (ACAM 44T), *F. gondwanense* (ACAM 49), *F. gondwanense* (ACAM 62), *F. gondwanense* (ACAM 46), *F. gondwanense* (ACAM 56), *F. salegens* (ACAM 48T), *F. salegens* (ACAM 52) and *F. salegens* (ACAM 54). See Appendix 2 for details on these strains.

The specificity of antibodies raised against the type strain of *H. subglaciescola* were tested by slide agglutination on that strain grown under different temperatures, salinities and light and on 1/2AOLPY agar and broth (Appendix 1.2.1). The standard conditions used were; 20 °C, 60 ‰ TDS, light, 1/2AOLPY broth. Only one condition was varied at a

time, conditions were varied as follows: temperature 5 °C, 15 °C, 30 °C; salinity 5 ‰ TDS, 30 ‰ TDS, 120 ‰ TDS; light and dark; 1/2AOLPY broth and agar.

3.2 Sample collection

Organic Lake, Ekho Lake, Fletcher Lake, Ace Lake and a coastal marine site were sampled every two months from May 1990 to January 1991 at the site of maximum depth using a perspex 2 l non-sterile flow-through sampler referred to as a Kammerer bottle. Samples were collected from within the oxylimnion, the oxycline and the anoxylimnion of the lakes and from a depth of 2 m from the coastal marine site. Six other lakes: Horse (now Williams) Lake, Johnston Lake, Straight-In (now Burch) Lake, Laternula Lake, Cemetery Lake and Deep Lake were sampled on single occasions (Figure 1.1; Table 1.1). Because of the possibility of diurnal variation within the water column (Pollard & Kogure, 1993) samples were usually taken around solar noon.

Two 10 inch diameter holes, separated by approximately 2 m, were bored with a Jiffy drill (Feldmann Engineering, Wisconsin). The first hole was used for measurement of temperature and density. Samples were taken from the second hole using a Kammerer bottle. In all cases depth was measured from water level rather than from the top of the ice. While lakes were unfrozen sampling was from the side of a boat. Samples were stored in 6 l polycarbonate autoclave bags tied at the top and transported in well insulated metal drums. Each bag was washed in 10% nitric acid, triple rinsed in double distilled 0.2 μm filtered water and autoclaved at 110 °C for 30 minutes before use. Radioisotope incorporation analysis commenced within 12 hours of sampling, other procedures following storage for up to 12 days, in the dark, at 1 °C.

After measuring the temperature at time of opening, aliquots were taken in the following order: (a) 20 ml sample into sterile glass bottle for inoculation of media; (b) 100 ml sample into sterile glass bottle for microscopy; (c) 2 x 100 ml samples into polypropylene containers washed in 10% nitric acid then triple rinsed in double distilled 0.2 μm filtered water for DOC analysis (one of which was frozen at -20°C); (d) 125 ml sample into glass reagent bottle for Winkler analysis; (e) 20 ml sample for the measurement of pH; and (f) 500 ml sample dispensed from a Schott bottle into individual containers for productivity analysis.

The remainder of each sample was filtered through a Whatman GF/C filter (GF/C; nominal 2-4 μm pore size) to provide a chlorophyll measure. Some of the GF/C filtered water was then used in the making of bacterial growth media.

3.2.1 Summary of sample dates

All grid references are from the map, 'Vestfold Hills, Princes Elizabeth Land' (1982). All dates in this thesis are in the form day/month/year.

Organic Lake was sampled on; 18/12/89, 16/6/90, 16/8/90, 12/10/90, 19/11/90, 8/1/91 and 5/3/91. During winter this site was accessed directly from Taynaya Bay at grid reference number LK853037. Sampling vehicles were taken along Highway Lake (grid reference number LK863027) through to Taynaya Bay. All samples were taken from the deepest point of the lake, located at the centre of the square described by grid reference number LK849035. This deep site is described in more detail in Gibson *et al.* (1989).

Ekho Lake was sampled on; 12/12/89, 22/12/89, 6/1/90, 12/2/90, 18/5/90, 14/7/90, 26/9/90, 1/12/90 and 18/1/91. Ekho Lake has a comparatively high water temperature (exceeded only by Anderson Lake) and supported all of the bacterial immunofluorescence study group. The lake was sampled with the greatest regularity and at ten depths rather than the normal five. During winter this site was accessed via a small unnamed freshwater lake (grid reference number LJ876964), across the pass directly below Lied Bluff and onto the north western beach of Ekho Lake (grid reference number LJ881967). In December 1989 a 42 m deep area was found by transect drilling (located at the centre of the square described by grid reference number LJ883965). All samples were taken from this deep point.

Fletcher Lake was sampled on; 17/12/89, 16/8/90, 12/10/90, 19/11/90 and 31/1/91. During winter this site was accessed directly from Taynaya Bay at grid reference number LK873039. Sampling vehicles were taken along Highway Lake (grid reference number LK863027) through to Taynaya Bay. While the lake was unfrozen, water flowed through this passage into Taynaya Bay. All samples were taken from the deepest point of the lake, located at the centre of the square described by grid reference number LK876041.

Ace Lake was sampled on; 1/5/90, 6/7/90, 20/9/90, 19/10/90, 19/11/90 and 15/1/91. During winter this site was accessed directly from Long Fjord at grid reference number LK850014. All samples were taken from the deepest point of the lake, located at the centre of the square described by grid reference number LK848028.

The seawater sample site was sampled on; 16/6/90, 22/7/90, 20/9/90, 29/9/90 and 27/1/90. This site, located directly west of the biology laboratory at Davis Station, was accessed by foot. The sample area was 10 m out from the shore in 3 m of water. All samples were taken from a depth of 2 m.

All miscellaneous lakes sites were sampled in the approximate middle of the lake. Burch Lake was sampled on the 16/8/90, Laternula Lake was sampled on the 19/8/90, Cemetery Lake was sampled on the 19/8/90 and Deep Lake was sampled on the 6/7/90.

3.3 Bacterial enumeration

For total counts, 4,6-diamidino-2-phenylindole (DAPI) was used to specifically stain double stranded DNA. Fluorescein isothiocyanate (FITC) conjugated anti-rabbit antibodies were used to stain those bacterial cells labelled with bacteria-specific rabbit antibodies. Optimum concentrations of stains and incubation times were determined empirically.

3.3.1 Total bacterial count

Samples used for microscopy were fixed with 0.5% gluteraldehyde at the sample site, stored in the dark at 1 °C and analysed within 12 days. Samples showed only slightly reduced counts after storage for 60 days. Each sample was incubated in the dark at room temperature with 0.1 μg ml^{-1} DAPI for 10 minutes. Sufficient sample, typically 0.2-2.0 ml, was then filtered through a Nuclepore polycarbonate filter (0.2 μm; 25 mm) to give approximately one hundred bacteria per field of view and the filter rinsed with phosphate buffered saline (PBS; Appendix 1.1.5). The filter was placed on a slide with one drop of phosphate buffered saline salts in glycerol (PBS salts in glycerol rather than water) and examined microscopically using a Leitz Laborlux 12 epi-fluorescent microscope with filter block A. Typically 5-8 fields of view were counted if the

count variation was ± 10%, if the variation was greater then more fields of view were counted.

3.3.2 Indirect immunofluorescence count

Fluorescence intensity decreases after exposure to light (fading) and can be influenced by pH. Samples were mounted in buffered glycerol, stored in the dark and exposed to the excitation light for as little time as possible (after Chantler & McIllmurray, 1987).

Each sample was incubated in the dark at room temperature for 20 minutes with 1.25×10^{-3} ml of antisera per ml of sample. Sufficient sample, typically 0.2-2.0 ml, was then filtered through a Nuclepore polycarbonate filter (0.2 μm; 25 mm) to give approximately one hundred bacteria per field of view and the filter rinsed with PBS. The filter was re-incubated in the dark at room temperature for 30 minutes with 2 ml of FITC conjugated 'anti-rabbit' antibody solution (Silenus; sheep raised anti-rabbit immunogloblin code RF) diluted to 1:1600 with PBS. The filter was then rinsed with PBS, placed on a slide with one drop of PBS-glycerol and examined microscopically using a Leitz Laborlux 12 epi-fluorescent microscope with filter block H2. Bacteria were identified on the basis of antigenicity and morphological appearance. Replicate samples were incubated with FITC conjugated 'anti-rabbit' antibody solution but without antibacterial antisera to determine background autofluorescent and non-specific staining.

3.4 Measurement of radioisotope incorporation

Tdr (1.08 mCi ml^{-1}, 45 Ci $mmol^{-1}$; Commissariat a l'Energies Atomique, France) stored in 70% ethanol and Leu (1.08 mCi ml^{-1}, 120 Ci $mmol^{-1}$; Commissariat a l'Energies Atomique, France) stored in 2% ethanol were held at 0 °C to minimise radiolysis (Ellenbroek & Cappenberg, 1991). Analytical grade thymidine and leucine used in isotope dilution analysis were obtained from Sigma Chemical Co., USA.

Radioisotope incorporation, isotope dilution and calibration of isotope concentration and incubation period were determined by the methods of Moriarty (1990). Incubation for 2 hr at *in situ* temperatures ± 1.5 °C with either 48 nM of Tdr or 9 nM of Leu was experimentally determined as suitable for all samples (indicated in Section 4.3.1). Samples with an *in situ* temperature below -1.5 °C were incubated at -1.5 °C.

Samples were incubated as soon as possible after sample collection in a clean non-reactive container and at *in situ* temperature, so that conditions were changed as little as possible. Oxic water samples (10 ml) were incubated in 30 ml polycarbonate bottles. Water samples from anaerobic depths (7.8 ml) were incubated with a 0.2 ml air gap in O-ring sealed 8 ml polycarbonate bottles. All samples were incubated in the dark. Radioactive substrate, non-radioactive substrate and stopper solution (Appendix 1.3) were introduced to the water sample in appropriate quantities depending on the experiment being undertaken and the bottle gently inverted several times. After incubation, 0.2 ml of stopper solution was added and the sample put aside for filtration. For blanks, stopper solution was added before the radioactive substrate.

Each sample was filtered through a 0.2 μm Nuclepore polycarbonate filter (held in a 12 cup Millipore sampling manifold). The filter was then rinsed twice with 5 ml of 80% ethanol (held at 0 °C), followed by five rinses with 1 ml of 5% trichloroacetic acid (held at 0 °C) (Appendix 1.3.2). The filter was then folded and placed in small polycarbonate vial with 2 ml of 5% trichloroacetic acid, boiled for 30 minutes and settled overnight. A 0.5 ml aliquot was dispensed into a 6 ml scintillation vial with 4.5 ml Optiphase 'highsafe' 3 (LKB). Chemiluminescence (CLM) in environmental samples was reduced by incubating sample vials in the dark at 25 °C for 24 hours then cooling to 1 °C before analysis. Though this procedure was derived empirically to reduce chemiluminesence, other researchers also leave their samples for 24 hours before analysis, presumably to overcome the same problem (Chrzanowski & Hubbard, 1988; Solic & Krtulovic, 1994). Radioactivity was measured with a RackBeta 1215 Liquid Scintillation Counter (LKB). Disintegrations per minute (DPM) were obtained from count per minute (CPM) data by the external standards ratio (ERS) method.

The utilisation of tritiated leucine to determine protein production involved a similar methodology to the thymidine techniques described in the last paragraph. However, the sample was filtered through 0.2 μm mixed cellulose filters and placed directly into the scintillation vials and 0.5 ml of ethyl acetate was added to dissolve the filter. The vial was shaken well with 4.5 ml Optiphase 'highsafe' 3 (LKB) and the radioactivity determined. No boiling was necessary.

DPM data from the scintillation counter was adjusted for isotope decay, radiolysis, specific activity and internal blanks. The mole isotope incorporation ml^{-1} h^{-1} (T) was calculated from DPM (Moriarty, 1990). An estimate of the rate of bacterial division h^{-1} (N) was determined from T with the theoretical conversion factor $5.1x10^{17}$ (Moriarty, 1990). Generation time was determined as the natural log of 2 divided by the specific growth rate ($N\ N_t^{-1}$). Where N_t was the total bacteria ml^{-1} as measured by vital staining with DAPI. Protein synthesis was calculated as Leu incorporation multiplied by 1797 (g protein h^{-1}) (Simon & Azam, 1989). These calculations are detailed in Appendix 3.

Data is presented as averages of duplicate or triplicate samples, subtracted against the average of duplicate blanks.

3.5 Measurement of dissolved organic carbon

DOC concentrations were measured using a pre-production model Skalar DOC Analyser (Analytical Technologies Australia, New South Wales). This unit digested DOC by acidification, chemical degradation (using persulfate) and strong ultra-violet light, quantitatively oxidising the DOC to carbon dioxide which was then reduced to methane and measured by flame ionisation detection (Hine & Bursill 1985). Chemical reagents were prepared according to the specifications supplied by Skalar and standard grade hydrogen, nitrogen and air (supplied by CIG) was used at specified pressures. DOC Analyser rinse water was double distilled water passed through a Millipore 'Norganic' organic scavenger resin cartridge. Samples were analysed following storage for up to 12 days in the dark at 1°C. Each sample set was processed against a glucose standard containing 10 mg of carbon per litre (mg C l^{-1}) dissolved in double distilled water passed through a Millipore 'Norganic' organic scavenger resin cartridge. When necessary, the sample was diluted before analysis to reduce the DOC concentration to within the range 0 - 10 mg C l^{-1}. Dilution was required for most of the samples from anaerobic waters.

Samples were analysed for total DOC after filtration through various Nuclepore polycarbonate filters (1.0 μm, 0.6 μm, 0.4 μm and 0.2 μm) to give DOC size fractions. Samples were filtered just prior to analysis. Before use, filters were rinsed with 15 ml of double distilled water.

Fresh sample was used for each filtration rather than filtrate from the last filtration.

After limited success with the computer and software provided by Scalar, a chart recorder was attached directly to the DOC Analyser DC output. This proved to be an excellent recording method, being much more sensitive than the original output device and allowing direct interpretation of results.

3.6 Measurement of other environmental variables

3.6.1 Oxygen

Oxygen concentrations were measured using standard Winkler titration methodology (Strickland & Parsons, 1972). It should be noted that the oxygen concentrations are approximate only, the sampling method involved some contact with the air. The full extent of this contamination was not determined as the known anaerobic areas (which would have been a good indicator) contained sulphides which reduce oxygen. Interference from iron, organics and nitrates also affect the accuracy of this method (Strickland & Parsons, 1972) and due to the extreme variance in the compositions of the lakes, these too were not determined.

Upon return to the laboratory a reagent bottle (air-tight) of known volume was gently filled with sample water. One ml of manganese chloride solution (Appendix 1.4.1) and 1 ml of potassium iodide solution (Appendix 1.4.2) were added with syringes. The vessel was capped and inverted several times, paying particular attention that there were no bubbles. The sample was then stored at room temperature until analysis. Continuing the analysis, a small amount of the sample was poured into a titration vessel and 1 ml of dilute sulphuric acid (Appendix 1.4.3) added to the reagent bottle to re-dissolve the manganese hydroxide floc. After gentle shaking the rest of the sample was poured into the titration vessel. Sodium thiosulphate solution (1:10 stock solution; Appendix 1.4.4) was titrated against the resulting solution using 1 ml of starch solution (Appendix 1.4.5) as an indicator.

A standard was analysed with each set of samples. The standard solution (Appendix 1.4.6) was diluted 1:2 with distilled water and 20.0 ml added to the titration vessel with 2 ml of acid solution. Sodium thiosulphate

solution was titrated against the resulting mixture using 1 ml of starch solution as an indicator. Dissolved oxygen concentration (corrected to 0 °C and 760 mm Hg) was calculated using the following formula :

$$[O_2]\ (ml\ l^{-1}) = (n \times f \times 0.05598) \div ((v - 2) \div 1000)$$

where **n = volume of sodium thiosulphate solution titrated against sample**
f = 20.00 ÷ (volume of sodium thiosulphate solution titrated against the standard)
v = sample volume (ml)

3.6.2 pH

Sample pH was measured with a MetroHM E604 pH meter.

3.6.3 Chlorophyll

Up to 4 l of sample was filtered through a GF/C filter which was dried, placed in 5 ml of 90% methanol then heated to boiling point in a water bath and boiled for 1 minute. The resulting solution, containing dissolved chlorophyll, was centrifuged at 3000 r.p.m. for 10 minutes and the liquid portion analysed using a Varian Techtron spectrophotometer. Optical density was measured at 665 nm and 750 nm against a 90% methanol blank. Chlorophyll concentrations were calculated in $\mu g\ l^{-1}$ using the standard formula of Talling (1974):

$$[Chl\ ]\ (\mu g\ l^{-1}) = 13.9 \times (O.D.\ 665 - O.D.\ 750) \times (v \div V)$$

where **v = volume of solvent (ml)**
V = volume of sample filtered (l)

Chlorophyll results were not corrected for phaeopigments.

3.6.4 Temperature and density

Initially, *in situ* temperature and density (at 20°C) were measured using a profiling DMA 35 density meter (manufactured by Anton PAAR, Austria). The instrument became erratic and then stopped working altogether over a period of three months from the middle of July 1989. The fault was due to the fracture and eventual corrosion of the joint

where the insulated cable met the measuring probe. Measurements during this time were corrected by calibration against the Kammerer bottle thermometer and sample density (*in situ*) measured in the field using a hand refractometer. After the middle of October 1989, sample density (*in situ*) was measured by hand refractometer. Figure 3.1 shows that the density measured by the hand refractometer was consistent with the profiling DMA 35 density meter. Temperature was measured using a thermistor type temperature probe (made by the Antarctic Division) calibrated against the Kammerer bottle thermometer.

All density readings were adjusted to their equivalent density at 20°C. The DMA 35 featured automatic temperature compensation; all hand refractometer results were adjusted to their equivalent density at 20°C (D_{20}) from the density recorded at *in situ* temperatures (D_T) (g ml^{-1}) according to an empirical formula modified from Gibson *et al.* (1990).

From experimental data of H. Burton (Pers. Comm., 1993) a relationship between density (g ml^{-1}) and salinity (‰) was derived and used in calculations:

$$\textbf{Salinity (‰)} = (D_{20} \times 1315.790) - 1313.421$$

This relationship was corroborated by S. Stark (Pers. Comm., 1994) (Figure 3.2):

$$\textbf{Salinity (‰)} = -2023.9 + 2667.6 \times D_{20} - 642.15 \times D_{20}^2$$

3.7 Bacterial reduction of DMSO and TMA*N*-O

The ability of a range of species from the ACAM collection to reduce DMSO (dimethyl sulphoxide) and trimethylamine *N*-oxide (TMA*N*-O) was tested following the methods of Oren & Trüper (1990) and using gas chromatography/mass spectrophotometry (GC-MS) for detection of reduction products. Type strains of the following species were tested under aerobic and anaerobic conditions; *Brevibacterium* sp. (ACAM 379; isolated in this study and described in section 5.8.1), *D. aquamarina, D. cupida, D. halophila, D. marina, D. pacifica, D. salina, D. venusta, H. elongata, H. halodurans, H. subglaciescola, Halovibrio variabilis*. Strains were grown in media and incubated at a temperature suggested by the culture collection from which the strains were

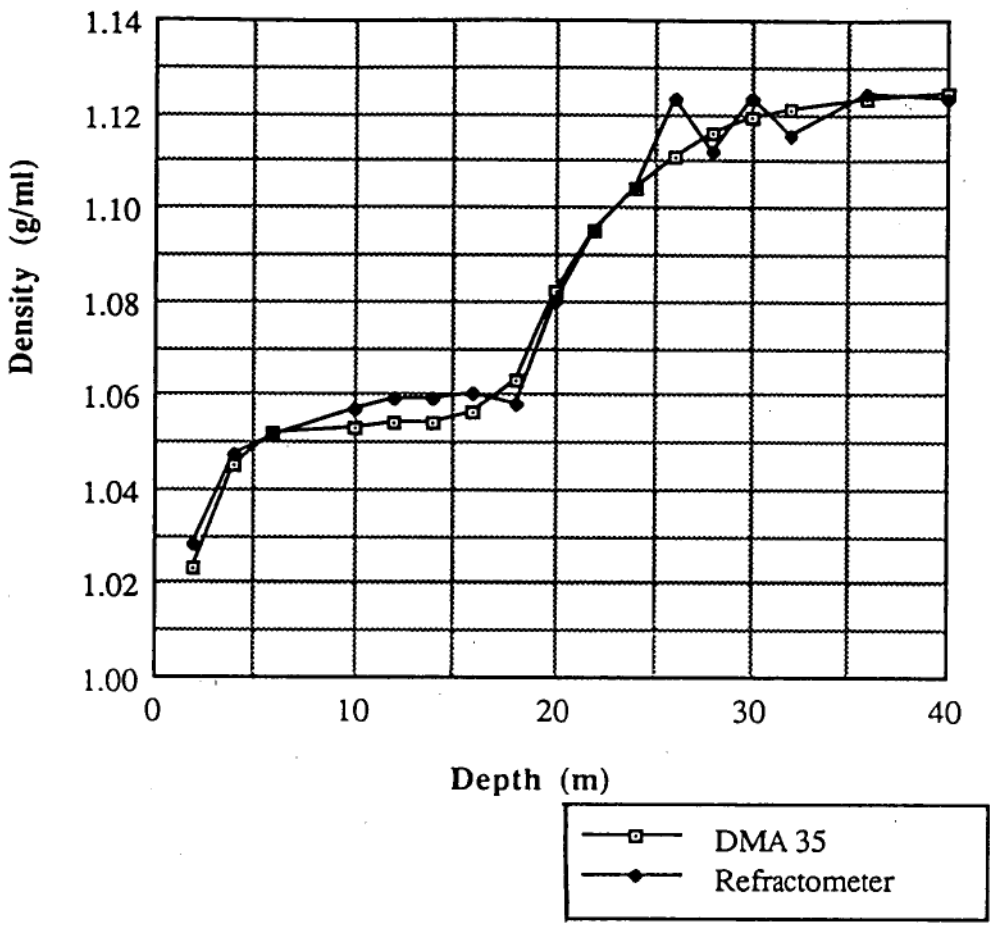


Figure 3.1: A comparison of densities (standardised to density at 20 °C) measured by a DMA 35 (PAAR) and a hand refractometer. Data from Ekho Lake, 22/12/1989.

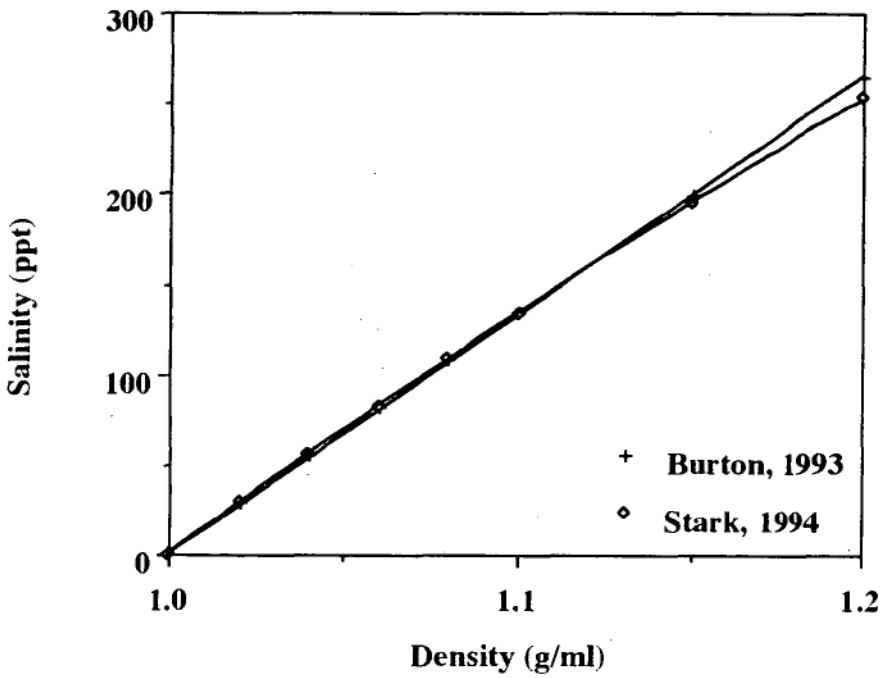


Figure 3.2: Salinity calculated from density at 20°C by the empirical formula of Stark (Pers. Comm., 1994) and Burton (Pers. Comm., 1993).

obtained. Growth medium were supplemented with 25 mM PIPES buffer (piperazine-*N*, *N*' -bis[2-ethanesulfonic acid]) and 3 ml l^{-1} DMSO or 3 g l^{-1} TMA*N*-O. Cells were incubated in 80 ml of growth media (boiled and purged with nitrogen) in 120 ml serum bottles under a gas phase of nitrogen. Tubes were sealed with rubber stoppers and aluminium crimp seals. Gas samples were taken by syringe through the rubber stopper.

3.8 Increase in tolerance to salt through uptake of proline

The ability of isolates from Ekho Lake to take up and utilise proline as a compatible solute was tested. Dilution plates of water from Ekho Lake were grown on nutrient agar (NA, Oxoid). Plates with well separated colonies were replica-plated using sterile velveteen. Plates of NA containing 0%, 5% and 8% NaCl and plates of NA containing 0%, 5% and 8% NaCl plus 0.2% proline (analytical grade) were used. Plates were incubated at 25 °C for 1 week. Those bacterial colonies growing on NA containing 5% or 8% NaCl plus 0.2% proline but not on NA containing just 5% or 8% NaCl were assumed to be using proline as an osmoprotectant.

3.9 Taxonomy of *Brevibacterium* sp.

3.9.1 Determination of mol % G+C

DNA preparation and analysis was modified from the original methods of Mesbah *et al.*. (1989). Their methods use high pressure liquid chromatography (HPLC) to determine mol% G+C and can be applied to very small amounts of cell material (i.e. a single colony). MilliQ water was used for preparation of reagents, which were autoclaved prior to use. Eppendorf tubes were used as reaction vessels throughout.

Two colonies were taken from an agar plate or slope and suspended in 50 μl of 0.03 M NaOH by vigorous vortexing. For Gram positive or resistant bacteria, 10 μl Lysozyme solution (0.04 g Lysozyme in 1 ml saline-EDTA; Appendix 1.1.6) was added and the mixture frozen with liquid nitrogen, thawed in hot water and incubated at 60 °C for an hour.

The method for the determination of mol % G+C by HPLC is detailed in Appendix 4.

3.9.2 Extraction of DNA for manual analysis of 16S r DNA

Bacterial cultures were grown in 500 ml Oxoid nutrient broth (no. 2) to the end of log phase. DNA was extracted by a modification of Marmur's method (Marmur, 1961), with the addition of a pronase treatment after the sodium dodecyl sulphate step modified from Blackall *et al.* (1985).

Cells were harvested by centrifugation and rinsed twice by suspension in saline-EDTA (Appendix 1.1.6) and re-harvesting. The resulting pellet was suspended in 8 ml of saline-EDTA. EDTA inhibits deoxyribonuclease activity which may degrade the DNA. This method is detailed in Appendix 5.

3.9.3 Determination of 16S rRNA sequence

The 16S rRNA gene from ACAM 379 was selectively amplified by the polymerase chain reaction (Saiki *et al.*, 1988) and sequenced. Dideoxynucleotide chain termination sequencing (Sanger *et al.*, 1977) adapted for double stranded polymerase chain reaction (PCR) product (Bachmann *et al.*, 1990) and further modified by Dobson (1993), was the basis of the determination of the 16S rRNA sequence. These methods were modified from Miller (1994) and Spielmeyer (1993). Every part of the gene was sequenced at least twice from one or both strands.

Equipment and kits were used according to the manufacturers instructions and were as follows;

GenClean II Kit (Bio 101 Inc., La Jolla, CA, USA)
Sequenase Version 2.0 Kit (USB)
LKB Macrophor Sequencing System (Pharmacia LKB)
LKB 2010-001 Macrophor apparatus (Pharmacia LKB)
LKB Multitemp II Thermostatic Circulator (Pharmacia LKB)
Bind-Silane and Repel-Silane (Pharmacia LKB)

The 16S rRNA sequence of ACAM 379 was aligned against the most closely related sequences taken in a pre-aligned form from the Ribosomal Database (GenBank; Olsen *et al.*, 1991). The ACAM 379 sequence was added and aligned using the HOMED (Homologous Sequence Editor) program (Peter Stockwell, NZ). Genbank was accessed

through ANGIS (Australian National Genomic Information Service - University of Sydney, NSW). This method is detailed in Appendix 6.

3.9.4 Analysis of menaquinones

Menaquinones were extracted and analysed using methods modified from R. Kroppenstedt (Pers. Comm., 1993) by P. Holloway (Pers. Comm., 1994). Up to 100 mg of freeze dried cells were placed in a clean dry screw cap test tube. The tube was wrapped in aluminium foil as isoprenoid quinones are susceptible to degradation by light. Two ml of methanol and 1ml of hexane were added to the tube which was sealed under a gas phase of nitrogen. The tube was shaken for 30 minutes then put into an ice bath until the two phases separated. The hexane phase was removed to a clean tube and 2 ml of ice cold hexane was added to the methanol phase. The hexane:methanol mixture was shaken for 5 minutes, 5 drops of sterile 0.3% (w/v) NaCl added then placed on ice until the two phases separated. The hexane phase was removed and combined with the first hexane fraction. The combined fraction was reduced by evaporation under a stream of nitrogen gas.

The combined hexane fraction (containing the lipoquinones) was dissolved in 25 μl of methanol:*tert* -butylmethylether (6:1) (v/v), spotted on a silica gel TLC plate and developed in methanol:*tert* -butylmethylether (6:1) (v/v). In the absence of ubiquinones (possible lipoquinone contaminates of menaquinone analysis) the sample (approximately 20 μl) was injected directly in to the HPLC and run through a Nova Pak C18, 3.9x150 mm, 4 μm packed column (Millipore) using methanol:dichloromethane (6:1) (v/v) as a carrier solvent. The absorbence was measured at 254 nm. All samples were run at room temperature (approximately 22 °C).

Lipoquinone retention time is affected by HPLC analysis methodology and the construction of the machine. A strain of known menaquinone composition, *Streptomyces griseus* (DSM 40 236) (Kroppenstedt, 1985), was used as a control and standard.

3.9.5 Determination of fatty acid profiles

Fatty acids were extracted and analysed using methods modified from R. Kroppenstedt (Pers. Comm., 1993) by P. Holloway (Pers. Comm.,

1994). Up to 20 mg wet weight of cells were placed in a clean dry screw cap test tube. Cells were lysed by the addition of 1 ml of a strong methanolic base (Appendix 1.5.1) followed by vortexing, heating in a boiling water bath for 5 minutes, vortexing and heating for a further 25 minutes. After cooling to room temperature, 2 ml of distilled water was added. An equal volume of Extraction solvent (Appendix 1.5.2) was added. After vortexing, the neutral lipid fraction was removed in the top solvent layer. This step was repeated three times. The remaining solution (containing fatty acid sodium salts) was acidified by the addition of 1 ml Acid solution (Appendix 1.5.3). An equal volume of methylation solvent (Appendix 1.5.4) was added. After vortexing, the fatty acid methyl ester fraction was removed in the top solvent layer. This step was repeated 3 times. The top solvent layer was dried under vacuum.

Fatty acid profiles were analysed by D. Nichols (University of Tasmania, Antarctic CRC) following the methods detailed in Nichols *et al.* (1993).

3.9.6 Growth relationships

All growth rates were determined using a SAS program to fit optical density data to the Gompertz function (program written by Glen McPherson of the University of Tasmania, Mathematics Department, 1990). Optical densities were measured during incubation using a Spectronic 20D spectrophotometer (Milton Roy Company). A Temperature Gradient Incubator Model TN-3 (Toyo Kagaku Sangyo Ltd.) was used to incubate samples over a range of temperature with gentle shaking.

Strains inoculated into Oxoid nutrient broth (no. 2) with 3% NaCl were incubated with gentle shaking at a gradient of temperatures from -1 to 46 °C for 4 days. The optical density of the culture was measured regularly during incubation and the growth rate determined from this data by the modified-Gompertz function (Gibson *et al.*, 1987). A minimum of 10-15 data points was used for each growth rate determination (McMeekin *et al.*, 1993b).

The square root of the rate of growth was plotted against temperature to determine the optimum temperature (T_{opt}) and extrapolated to estimate

the nominal minimum and maximum temperatures for growth (T_{min}; T_{max}) using the four parameter square root model of Ratkowsky *et al.* (1982).

Strains inoculated into Oxoid nutrient broth (no. 2) with a final salt concentration from 0.4 (salinity of Oxoid Nutrient Broth) to 30% or into AOLPB (Appendix 1.2.1) were incubated with gentle shaking at 24 °C for 3 days. The rate of growth was adjusted to rate of growth at 30 °C using the nonlinear equation of temperature relations determined above. Salinity was converted to a_w by an empirical formula derived from data presented by Chirife and Resnik (1984):

$$\mathbf{a_w = 9.985x10^{-1} - 4.630x10^{-3} \times \%NaCl - 1.689x10^{-4} \times \%NaCl^2 \quad (r^2=1.0)}$$

The rate of growth at 30 °C was plotted against a_w then converted to determine the optimum %NaCl ($Salts_{opt}$) and extrapolated using a linear equation to estimate the maximum %NaCl for growth ($Salts_{max}$).

Strains inoculated into nutrient broth (NB; Oxoid) with 3% NaCl adjusted to pH 3.5 to 9.1 (measured after autoclaving) using 0.25 M HCl and 0.1 M NaOH were incubated with gentle shaking at 24 °C for 3 days. NB with 3% NaCl had a pH of 7.6 after autoclaving. The rate of growth was adjusted to rate of growth at 30 °C using the nonlinear equation of temperature relations determined above.

3.10 Analysis of data

All contour plots were created using **ncar**, a VAX graphics package. Mark Conway (Antarctic Division, Systems Programmer) wrote the FORTRAN interface that set parameters and allowed data input from a Macintosh computer. Other work was processed on a Macintosh computer using Microsoft Excel (Microsoft Corporation), Microsoft Word (Microsoft Corporation), Microsoft Works (Microsoft Corporation), Systat (Systat Inc.) and Cricket Graph (Computer Associates Intl.) software packages. Surface response plots were created using Systat.

4. RESULTS

4.1 Physical characteristics of the lakes

The surface of all five of the study sites was frozen over the winter and maintained ice cover approximately 1.5 m thick until midsummer. With the summer thaw, the upper 4 to 5 m of each of the lakes mixed and became less saline. All the study lakes were meromictic; permanently stratified with lighter, less saline water overlying a more saline layer (Table 1.1). This stratification leads to anoxia in the lower layer through bacterial action over long periods of time. In all the lakes studied, the oxycline remained stable during the year. The salinity and temperature throughout the water column in each lake during January 1991 can be compared in Figures 4.1 and 4.2.

4.2 Direct enumeration of microbiota by immunofluorescence microscopy

4.2.1 Specificity of the antisera

Antibodies raised against the type strains of *H. subglaciescola* and *F. gondwanense* were strain specific. Antibodies raised against the type strain of *H. meridiana* were species specific. Antibodies raised against the type strain of *F. salegens* cross-reacted outside the species. The specificity of antisera raised against *H. subglaciescola* and *H. meridiana* is shown in Figure 4.3. The dendrogram shown in Figure 4.3 was adapted from numerical analysis of 134 physical characteristics and shows phenotypic similarity between strains (James *et al.*, 1990).

No reactivity was observed between the antisera and other non-target bacterial species with the exception of the marine bacterium, *D. venusta* (DSM 4743) which demonstrated a strong agglutination reaction with antisera raised against the type strain of *F. salegens*. Slide agglutination experiments demonstrated that the type strain of *H. subglaciescola* was antigenically similar and showed no decrease in antibody specificity when grown under different temperatures, salinities, light conditions or on agar or broth.

Some of the antisera demonstrated low activity and were unsuitable for experimental use. Each batch was investigated individually before use.

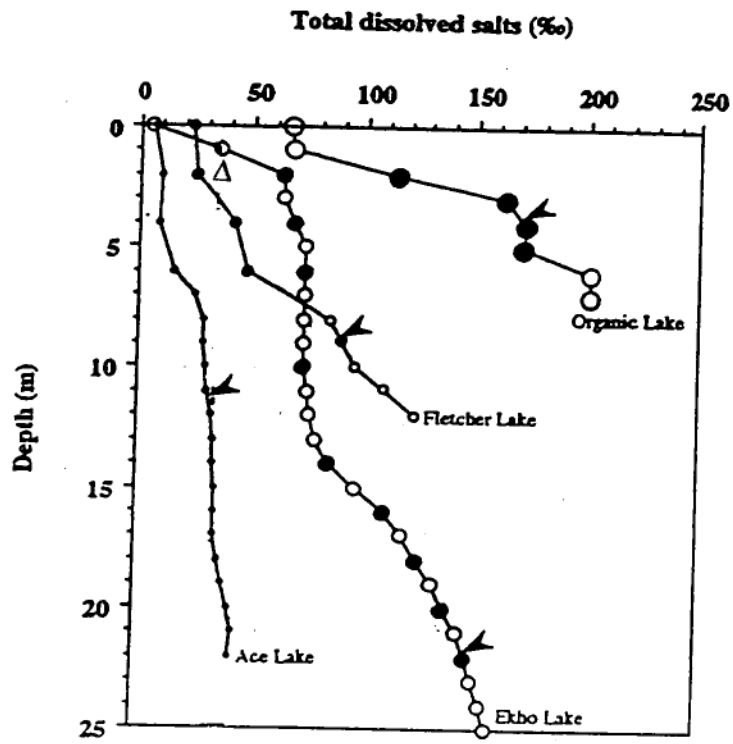


Figure 4.1: Total dissolved salts (TDS) at all sites in January 1991. The coastal marine site was sampled at 2 m depth only for which TDS is indicated by (Δ). The oxycline is indicated by arrows. Depths where samples were taken are indicated by closed circles.

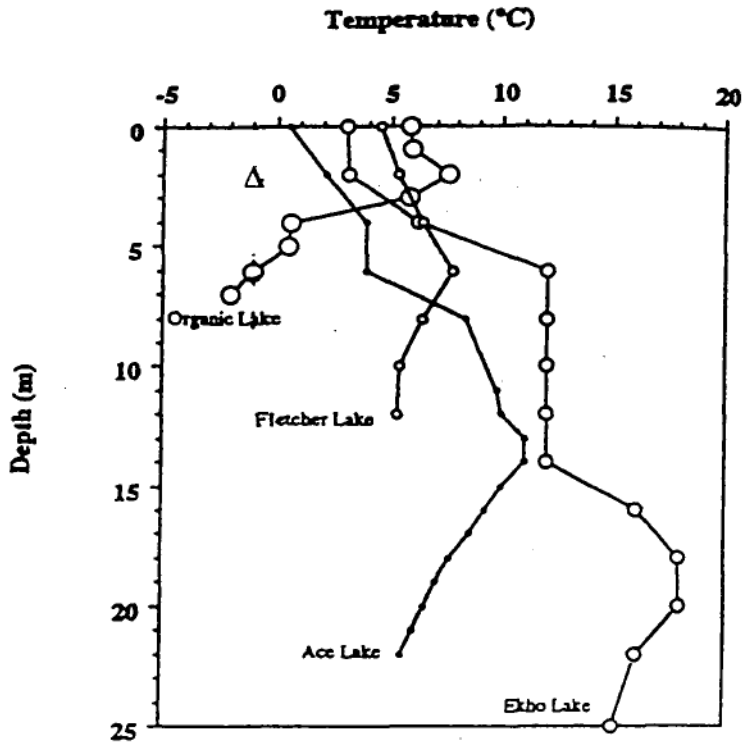


Figure 4.2: Temperature at all sites in January 1991. The coastal marine site was sampled at 2 m depth only for which temperature is indicated by (Δ).

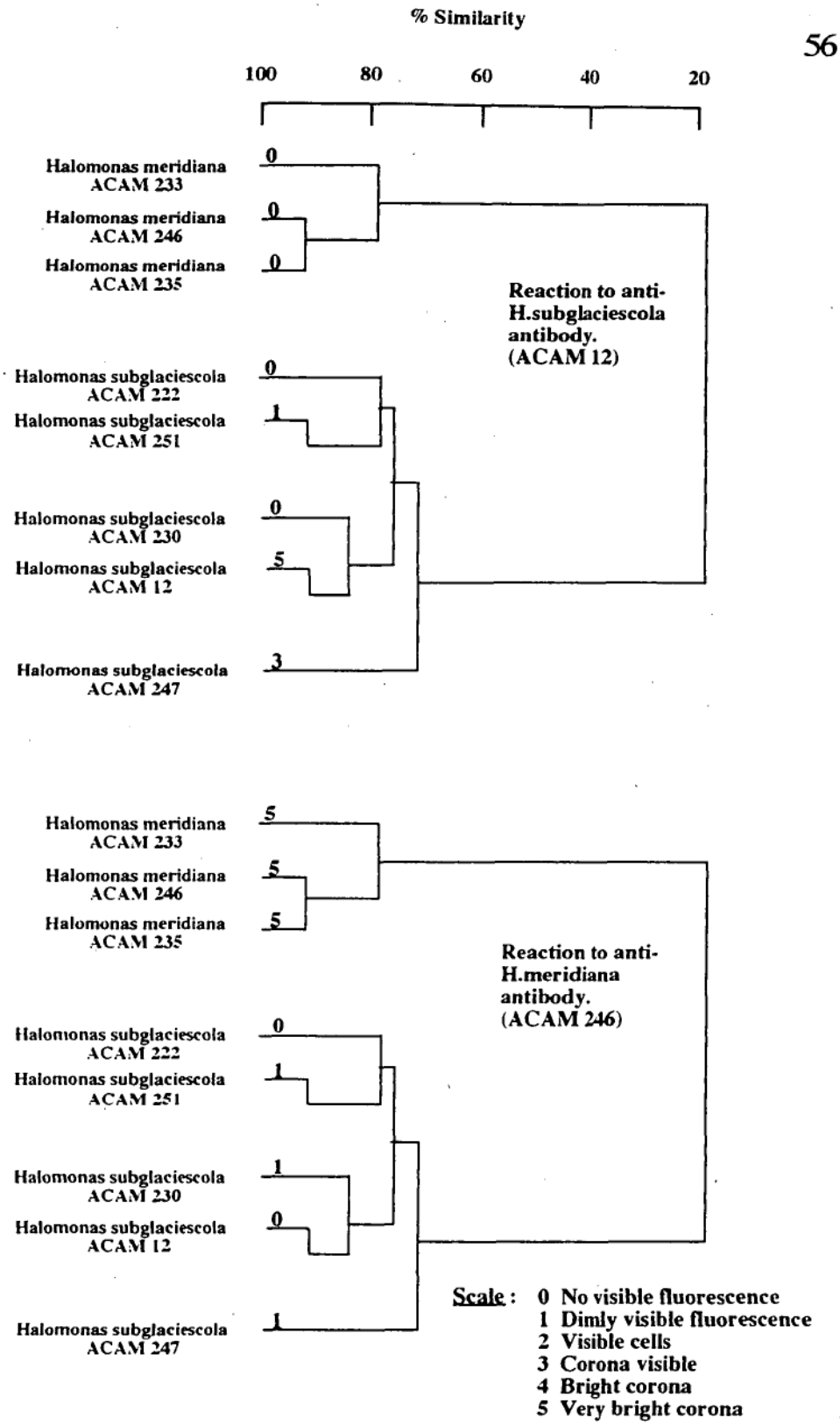


Figure 4.3: Reaction by immunofluorescent microscopy of *Halomonas* spp. to antibodies raised against *H. meridiana* and *H. subglaciescola*. The dendrogram was adapted from James *et al.* (1990) and shows phenotypic similarity calculated from numerical analysis of 134 physical characteristics.

All samples were fixed with 0.5% gluteraldehyde and showed slightly reduced activity after 60 days. The addition of 0.2 ml of gluteraldehyde (25% in water) per 10 ml of sample was compensated for in the calculation of bacterial concentration (total bacteria ml^{-1}). Mixed cellulose and glass fibre filters and any filters stained with irgalan black demonstrated prohibitively high background fluorescence. Nuclepore polycarbonate 0.2 μm filters showed little fluorescence and were adequate for immunofluorescent work. Precipitation of solids in anaerobic samples sometimes made counting difficult.

4.2.2 Presence of test species

Up to 2% of the total bacterial population of the lakes were autofluorescent or stained non-specifically. Background counts were accounted for in immunofluorescence counts. Known quantities of the four Antarctic bacteria used to raise the antisera (suspended in PBS) were used as internal standards in the immunofluorescence counts. At cell concentrations above $2x10^3$ cells ml^{-1}, recovery rate was more than 95%. When analysing samples, no attempt was made to count fluorescent cells at concentrations below $2x10^3$ cells ml^{-1}, these data were given the value 0 cells ml^{-1} when presenting the results on surface response curves (Figure 4.4; Figure 4.5). The *F. salegens* serogroup was always less than 2% of the total bacterial count and was not plotted. Data for this group is presented in Appendix 7. The surface response curves were produced using the mathematical program Systat (Systat Inc.). Surface plots were smoothed by the distance weighted least squares method. This method produced a locally weighted surface with each point on the surface calculated by four weighted multiple regressions allowing the surface to flex locally to fit the data better. The method gave a smooth surface through the data set, emphasising broad trends. Areas with sparse data points or with smaller features (valleys or peaks) were interpreted with caution.

From August 1990 all four serogroups were observed in Organic Lake and Ekho Lake and to a small extent in Fletcher Lake, but not in Ace Lake or the local seawater. The *H. meridiana*, *H. subglaciescola* and *F. gondwanense* serogroups occurred at discrete locations within the water column. In Organic Lake, *F. gondwanense* was found most often at 2 m depth and *H. subglaciescola* at 3 m depth from October 1990 till the end of the sampling period (Figure 4.4). In Ekho Lake, serogroup

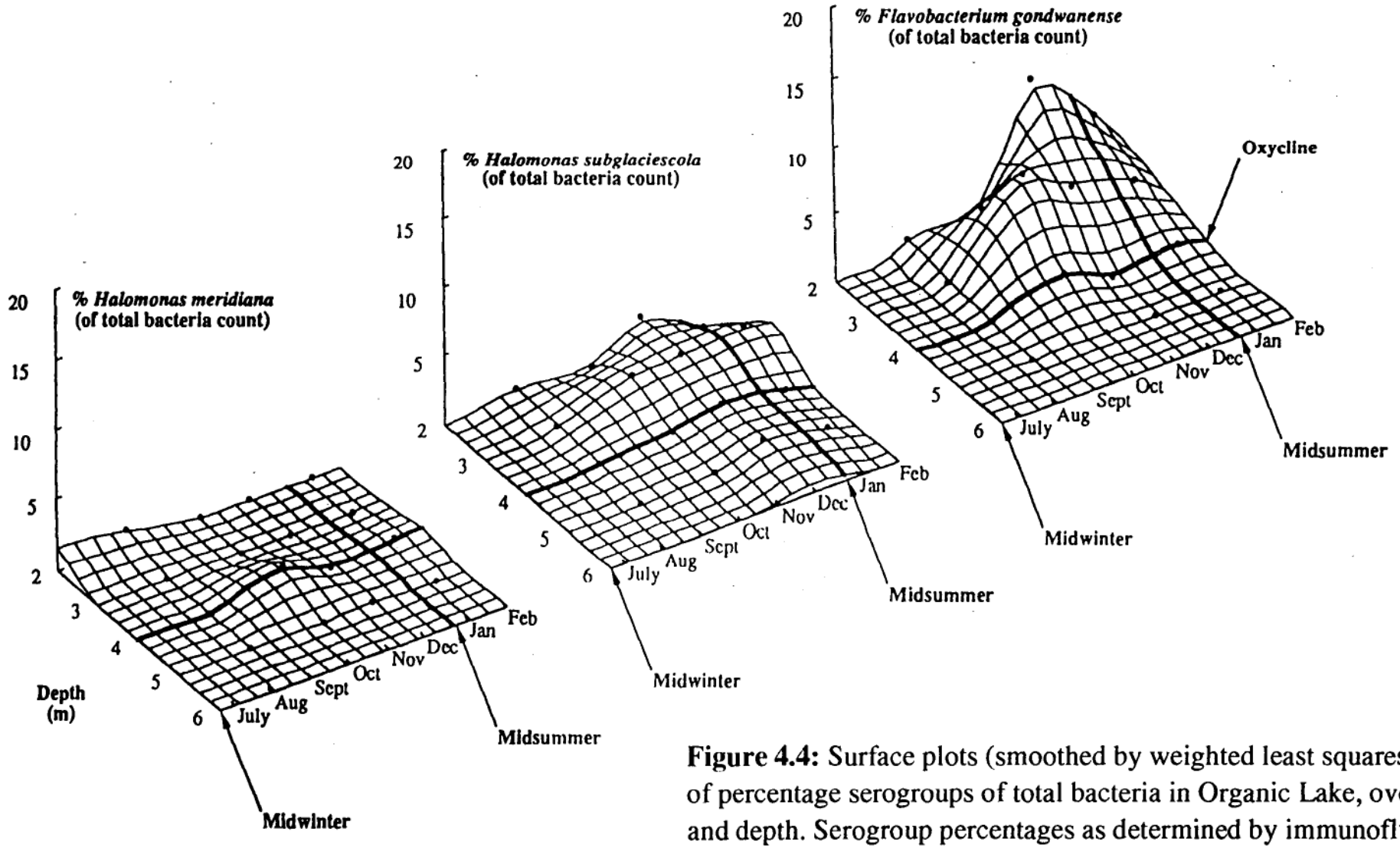


Figure 4.4: Surface plots (smoothed by weighted least squares method) of percentage serogroups of total bacteria in Organic Lake, over time and depth. Serogroup percentages as determined by immunofluorescent staining. Four depths were sampled at intervals of two months.

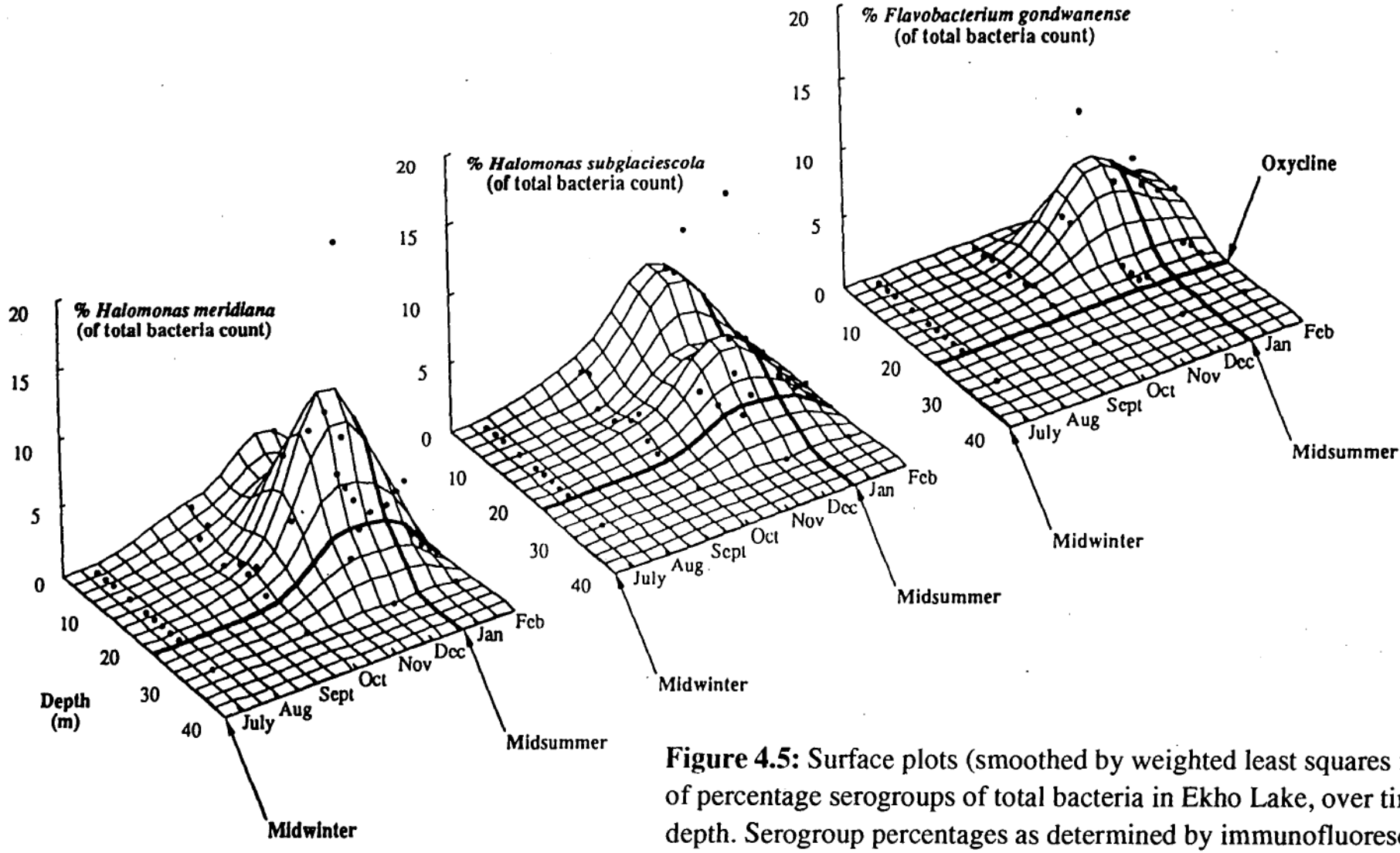


Figure 4.5: Surface plots (smoothed by weighted least squares method) of percentage serogroups of total bacteria in Ekho Lake, over time and depth. Serogroup percentages as determined by immunofluorescent staining. Ten depths were sampled at intervals of two months.

abundances peaked in December 1990, declining to January 1991. The *H. subglaciescola* serogroup was found most often from 2 m to 6 m depth, *F. gondwanense* from 6 m to 14 m depth and *H. meridiana* from 10 m to 18 m (Figure 4.5).

Peak percentages of the four serogroups, sample depth, date, total bacteria, DOC, TDS and temperature in Organic Lake, Ekho Lake and Fletcher Lake are shown in Table 4.1. Fletcher Lake contained less than 4% of all four serogroups and showed no seasonal pattern. All samples from Ace Lake and local sea water contained less than 0.1% of the four serogroups and showed no seasonal pattern. Small populations of the three main serogroups occurred up to 2 m below the oxycline in Organic Lake and Ekho Lake (Figure 4.4; Figure 4.5). As all bacteria shown to react to the four antibodies were aerobes (Dobson *et al.* 1991, Franzmann *et al.* 1987b, James *et al.* 1990), these populations were probably comprised of cells settled from the upper waters. Data values are detailed in Appendix 7.

H. subglaciescola was originally isolated and characterised by Franzmann *et al.* (1987b) from Organic Lake. This species was also isolated from a number of Vestfold Hills saline lakes by Garrick & Gibson (1988). Franzmann *et al.* (1987b) characterised 29 strains of *H. subglaciescola* from Organic Lake and James *et al.* (1990) characterised a further 20 strains. Immunofluorescence indicated that the single strain ACAM 12 comprised a considerable proportion of the total bacterial population of Organic Lake, Ekho Lake and Fletcher Lake. It is probable that the species *H. subglaciescola* comprised a much larger part of the bacterial biota of these lakes. Similarly, the antisera used to detect *F. gondwanense* was strain specific and only indicative of the presence of one of a number of strains of *F. gondwanense*. Dobson *et al.* (1991) characterised 10 strains of *F. gondwanense* from Organic Lake alone. It is probable that *F. gondwanense* comprised a larger part of the bacterial population of Organic Lake, Ekho Lake and Fletcher Lake than indicated by immunofluorescence.

The antisera raised against *H. meridiana* were species specific, the populations of *H. meridiana* were probably as indicated by immunofluorescence.

Table 4.1: Peak percentage of total bacteria of the four bacterial serogroups at the three most saline sites.

			Site data specific to sample depth					
Serogroup	Site	Peak % of total	Sample date	Depth (m)	Total bacteria (cells ml^{-1})	DOC (mg C l^{-1})	Salinity (‰)	Temp. (°C)
H. meridiana	Organic L.[a]	2	Oct. 1990	4	$2x10^7$	39.0[d]	165	-5.6
	Ekho L.[b]	23	Dec. 1990	16	$8x10^5$	4.9	83	15.6
	Fletcher L.[c]	3	Oct. 1990	2	$3x10^6$	1.0	61	-3.1
H. subglaciescola	Organic L.	3	Jan. 1991	3	$1x10^7$	30.1	160	5.8
	Ekho L.	16	Dec. 1990	16	$8x10^5$	4.9	83	15.6
	Fletcher L.	2	Oct. 1990	4	$3x10^6$	0.9	62	-3.1
F. gondwanense	Organic L.	10	Nov. 1990	2	$6x10^6$	24.5	146	-8.4
	Ekho L.	9	Dec. 1990	6	$1x10^6$	2.0	65	11
	Fletcher L.	2	Aug. 1990	2	$4x10^6$	1.0	56	-2.0
F. salegens	Organic L.	<1	Oct. 1990	3	$6x10^6$	24.5[d]	163	-10.1
	Ekho L.	2	Sept. 1990	14	$2x10^6$	4.1	83	15.9
	Fletcher L.	1	Nov. 1990	6	$8x10^6$	2.5	74	2.7

[a] Organic lake was 7 metres deep with an oxycline at 4 metres.

[b] Ekho lake was 42 metres deep with an oxycline at 22 metres.

[c] Fletcher lake was 12 metres deep with an oxycline at 8 metres and is subject to periodic seawater incursions.

[d] DOC levels were not determined during October 1990 in Organic Lake, results shown from November 1990.

F. salegens was originally isolated from Organic Lake and characterised by Dobson *et al.* (1991). Immunofluorescence cross-reactivity data showed that the *F. salegens* serogroup included *D. venusta.* The bacterial biota of the saline lakes of the Vestfold Hills is probably of marine origin (James *et al.*, 1990) so it is conceivable that *D. venusta*, a halophilic marine bacterium, should be present. Though no worker has found *D. venusta* in any lake of the Vestfold Hills or Antarctica, cell counts for the *F. salegens* serogroup were deemed less useful than for the other three serogroups. The *F. salegens* serogroup represented only a small proportion of the total count in all lakes and was always less than 2% of the total bacterial count (Table 4.1).

Data for lakes sampled on only one occasion are presented in Appendix 7. Only *Flavobacterium* sp. was found in significant numbers. Populations of *Halomonas* sp. were present in Laternula Lake and Straight-In (Burch) Lake but were <0.3% of total bacterial count.

Analysis of mixed cultures grown from Organic Lake water inoculated PYVG broth (Appendix 1.2.4) showed 1 in 10 bacterial cells reacted to *H. subglaciescola* antisera. No other serogroups were detected in Organic lake enrichment and none of the species present in this enrichment were present in enrichments of any of the other lakes. Under microscopic examination the Organic Lake enrichment consisted of similar large cells typical of *H. subglaciescola*; the non-reactive cells seen under fluorescence were probably a different strain of *Halomonas* sp..

Forty three bacterial isolates from Ekho and Organic Lake (white, off white, yellow and orange) were grown on 1/2 strength AOLPYA (Appendix 1.2.1), suspended in 1/2 strength AOL salts (1/2 strength AOLPYA without the peptone, yeast or agar) and tested by agglutination against the four serogroups. Of these isolates, 27 were seropositive indicating that the majority were from the genera *Halomonas* or *Flavobacterium.* Colony formation and cellular morphology supported this indication.

4.2.3 Distribution of total bacteria

Background fluorescence was easily distinguishable from DAPI stained bacteria. Though species composition changed markedly over time, total

bacterial numbers in the aerobic layers of the lakes remained relatively constant and decreased only slightly over the period of this study. Bacterial numbers were generally two to five times greater around the oxycline and in the anaerobic waters of the lakes than bacterial numbers in the aerobic waters.

In Organic Lake, Ekho Lake, Ace Lake and the local seawater, there was a substantial increase in bacterial numbers just below the oxycline soon after the return of light, during early summer. Fletcher Lake was not sampled at this time. Fletcher Lake show an increase in bacterial numbers at the oxycline in January which the other sites did not share. Isopleths of Ekho Lake bacterial totals over time are shown here as an example (Figure 4.6). Data from the other sites are detailed in Appendix 7.

4.3 Measurement of microbial activity by radioisotope incorporation

4.3.1 Determination of optimum experimental conditions

Isotope incorporation calibration experiments were carried out to determine optimum experimental conditions. Because of the inherent variability of parameters within and between Antarctic lakes and other non-Antarctic environments, the calibration experiments were conducted at all sites at various times over the sampling season and at various depths.

The routine data obtained from standard analysis is presented as DPM directly converted from CPM, ESR and CLM data. A direct relationship was found between CPM vs. CLM (Figure 4.7) so that even those results with CLM values over 1% were used. Throughout this thesis, data sets were graphed and fitted with polynomials. Though the polynomials may not be indicative of the relationships between the data sets, they were included for convenience of interpolating the data points and as a measure of the interdependence of the data. Though both single channel ratio (SCR) and ESR measurements were taken for each sample, only the ESR results were used. SCR readings showed greater total variability. The quenching curve for ESR is shown in Figure 4.8. Various formula and correction factors were used in the calculation of radioisotope incorporation results and the conversion of raw

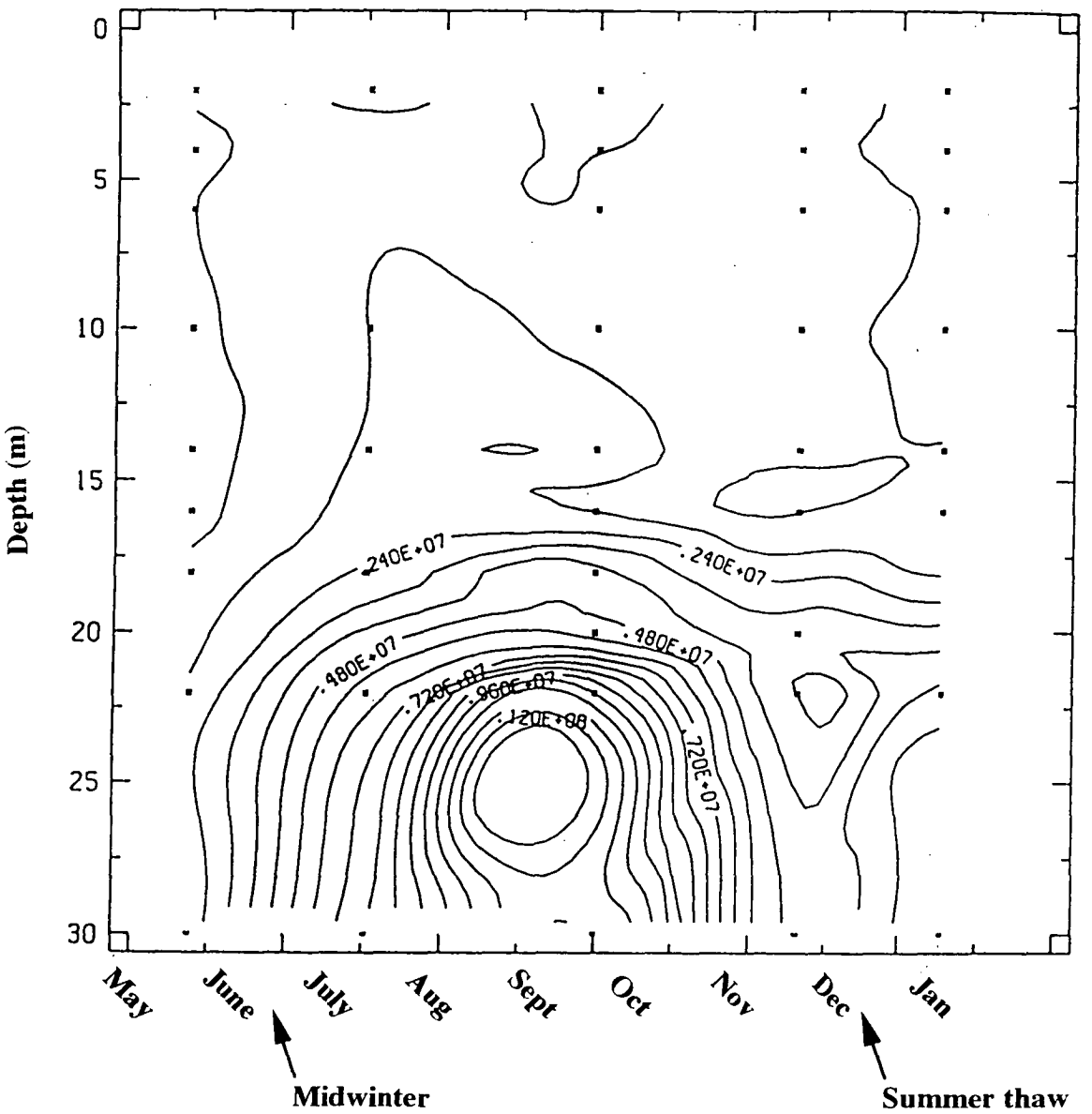


Figure 4.6: Isopleths of Ekho Lake total bacteria ml^{-1} versus depth and date. On the date axis, midwinter was day 204 and midsummer day 387. The highest number of bacteria occurred just below the oxycline at the end of winter.

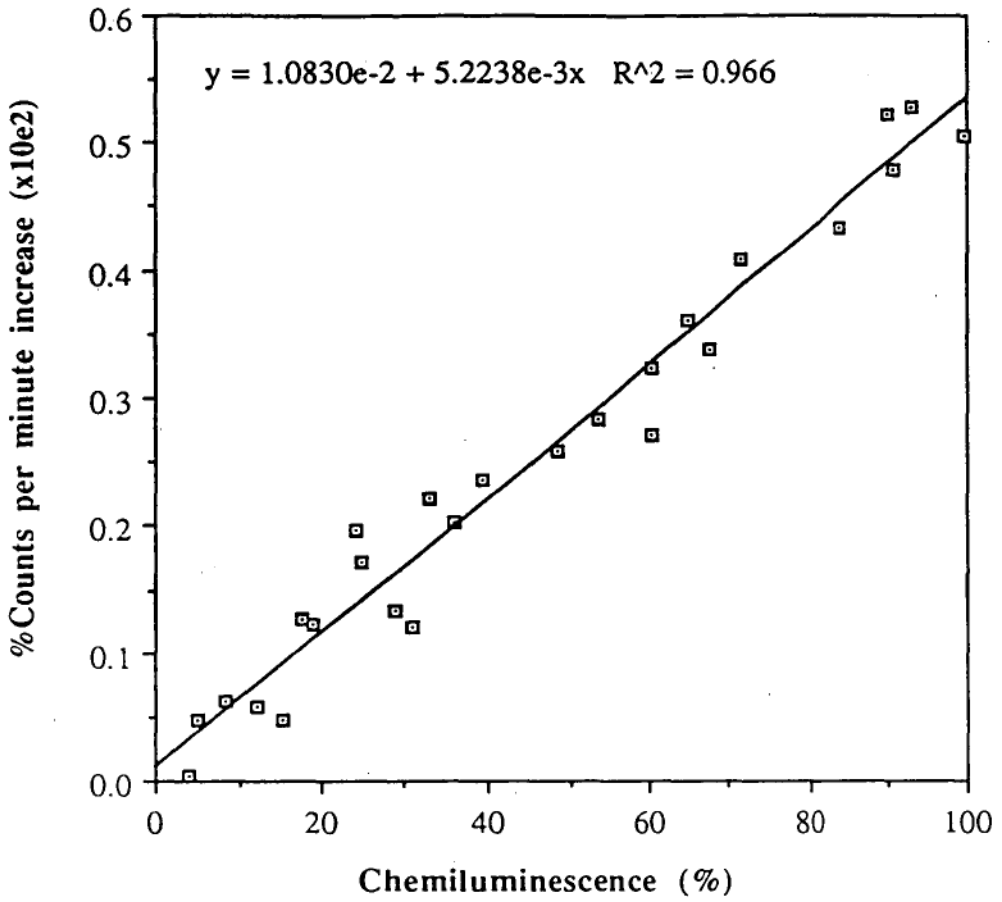


Figure 4.7: Increase in % counts per minute versus chemiluminescence. Plotted data is from both Single Channel Ratio and External Standard Ratio counts.

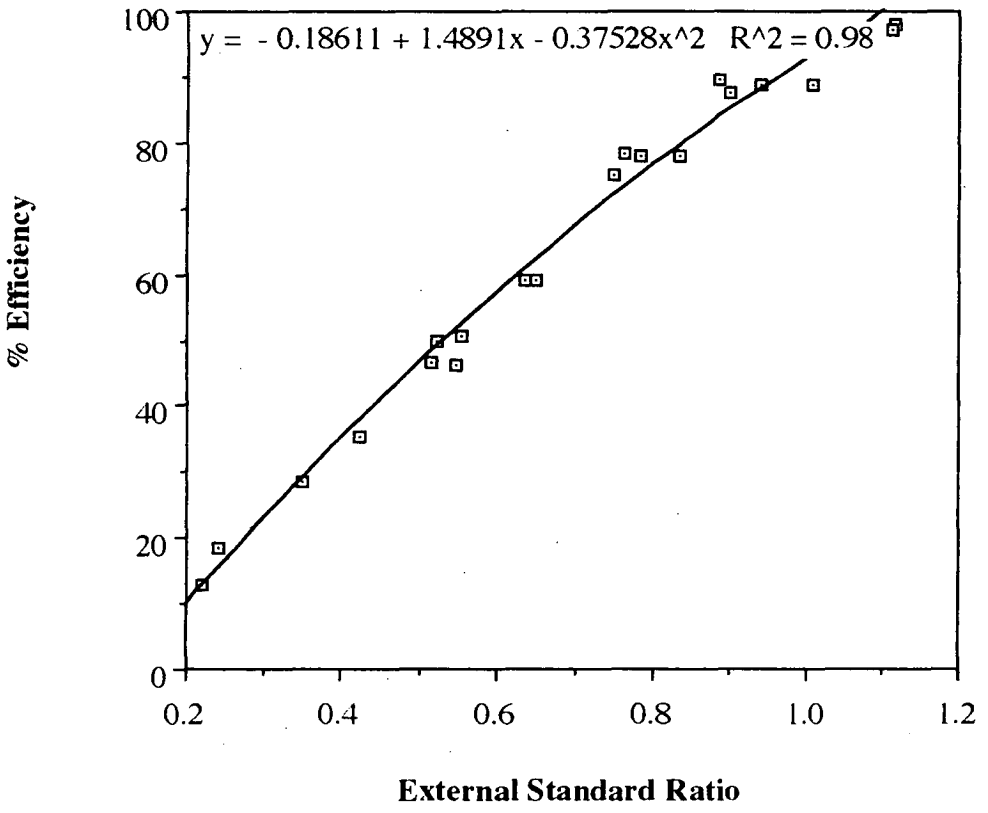


Figure 4.8: Quenching curve for External Standard Ratio results. Quenching expressed as % Efficiency.

radioisotope incorporation data. These formula are detailed in Appendix 3.

Of fifteen time course studies which analysed both Tdr and Leu incorporation, eleven showed that isotope incorporation increased linearly with time for at least three hours (r^2 values ≥ 0.8). Two studies from anaerobic waters showed results with no discernible trend possibly due to the inability of anaerobic bacteria to incorporate Tdr (see Section 5.3.2.2). The Ekho Lake time course study for January, 4 m depth, is shown here as an example (Figure 4.9). A surface response curve of bacterial number ml^{-1} h^{-1} versus depth and incubation time for Ace Lake in January is shown in Figure 4.10. Data from the other sites are detailed in Appendix 7.

Radioisotope incorporation increased with increasing radioisotope addition until a maximal incorporation value was reached. This saturation point varied over season, depth and site. All sites, at various depths and times, showed almost complete saturation of Tdr incorporation after the addition of at least 48 nM of Tdr (11 studies). Ekho Lake incorporation studies for January, 4 m depth are shown here as an example (Figure 4.11). Data from the other sites are detailed in Appendix 7. All sites investigated showed saturation of Leu incorporation after the addition of approximately 46 nM of Leu (3 studies). Calculation of maximum incorporation velocity by the method of van Looji and Riemann (1993), indicated the standard addition of 9 nM Leu underestimated incorporation of Leu by approximately 2 - 3 times (Figure 4.12). These calculations were used to correct Leu incorporation velocity to reflect a saturated system. Other authors have used Michaelis-Menten kinetics to estimate V_{max} (Kirchman *et al.*, 1986; Servais, 1995), insufficient data were collected to use this method for Leu incorporation in this study. Calculations using Michaelis-Menten kinetics indicated that in Figure 4.11 Tdr incorporation was 95% saturated after the addition of 48 nM of Tdr. Tdr incorporation results were not corrected to reflect a fully saturated system.

The validity of incorporation of Tdr and Leu values was examined using isotope dilution experiments (11 studies). Incubation with a constant level of isotope while varying the total concentration of the substrate (thymidine or leucine) gave an indication of the exogenous substrate pool. A linear regression of the inverse of DPM vs. total substrate

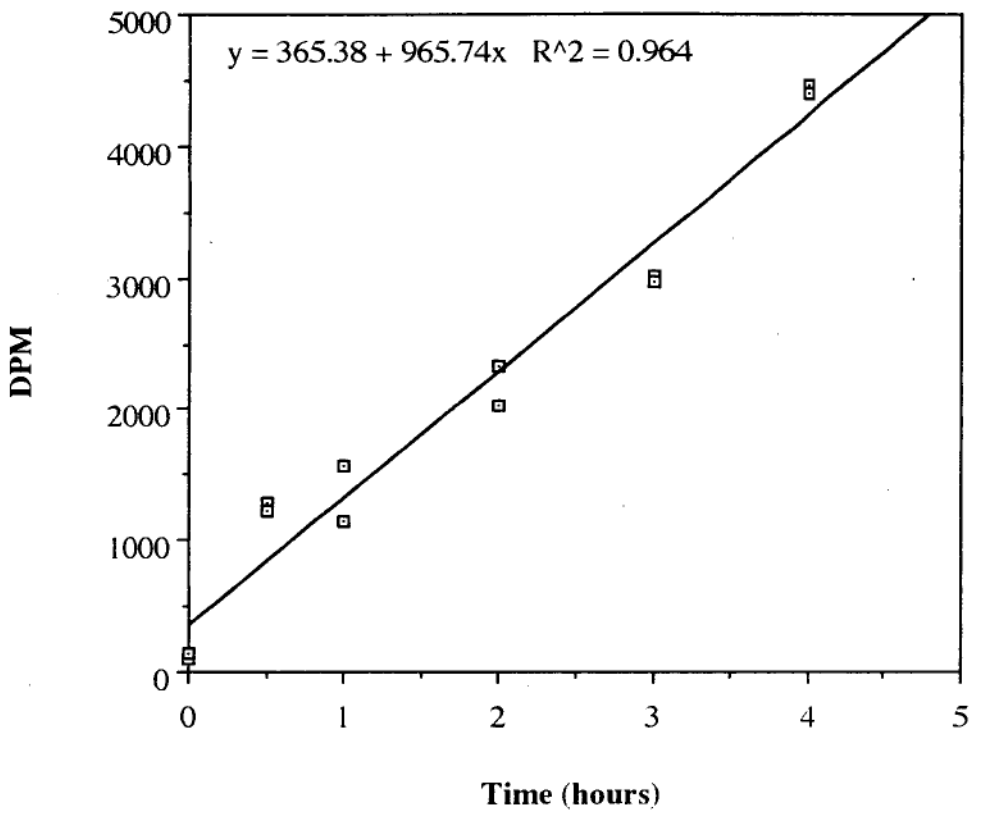


Figure 4.9: DPM of Tdr incorporation experiment for Ekho Lake 4 m depth (8/1/1991) versus incubation time.

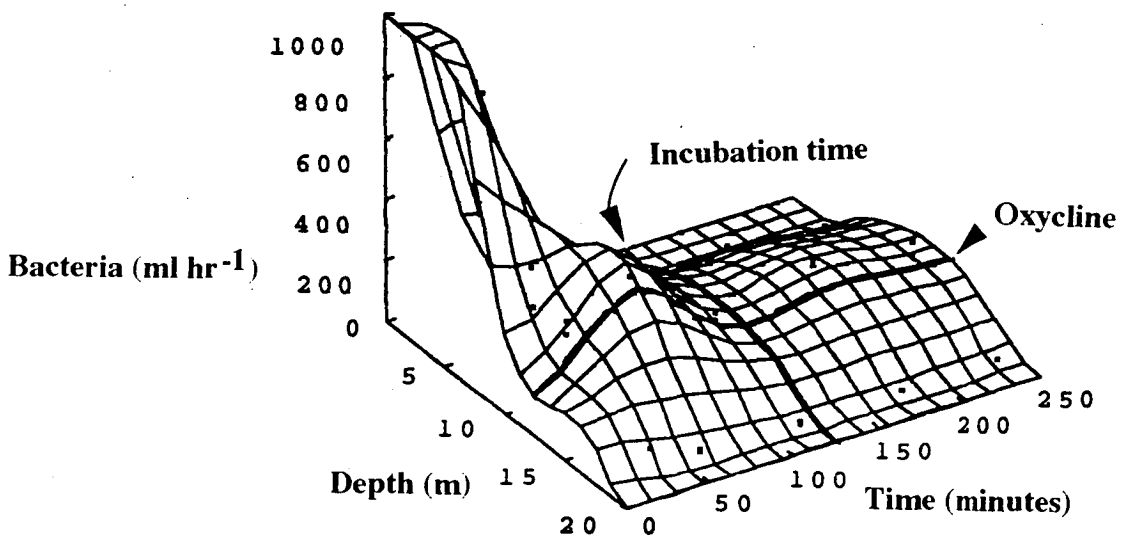


Figure 4.10: A surface plot (smoothed by weighted least squares method) of bacterial number ml^{-1} h^{-1} calculated by Tdr incorporation versus depth and incubation time. The oxycline is marked as is the incubation time (120 minutes) chosen for routine analyses.

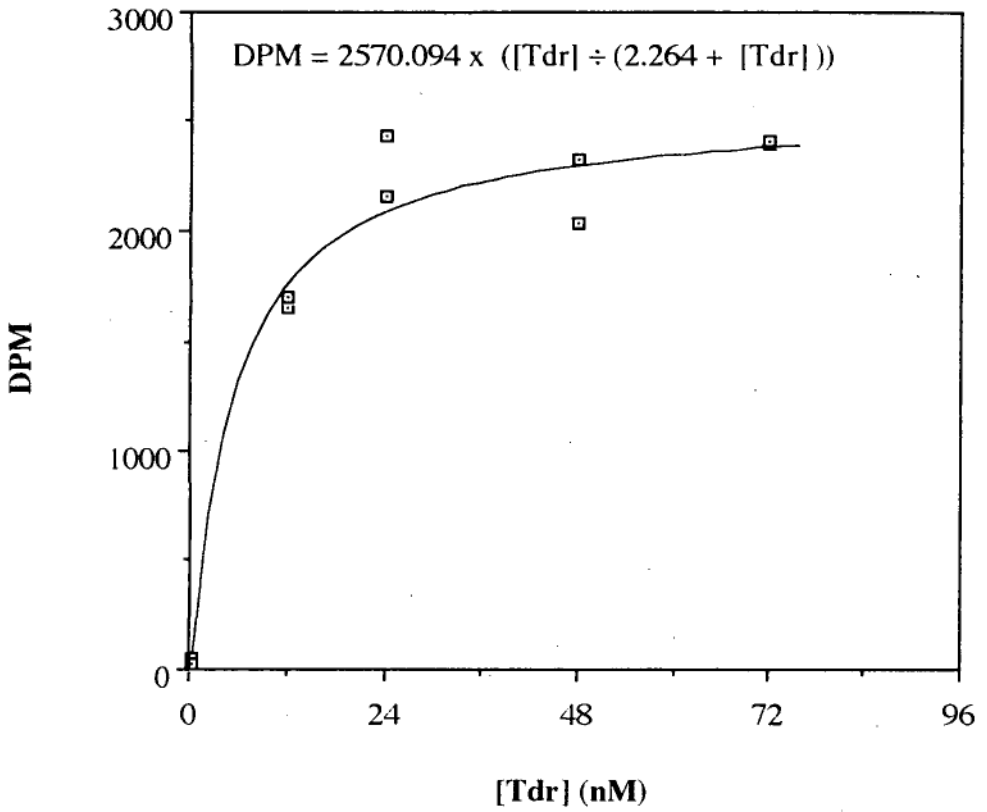


Figure 4.11: DPM of Tdr incorporation experiment for Ekho Lake 4 m depth (8/1/1991) versus Tdr concentration. Plotted line described by Michaelis-Menten kinetics.

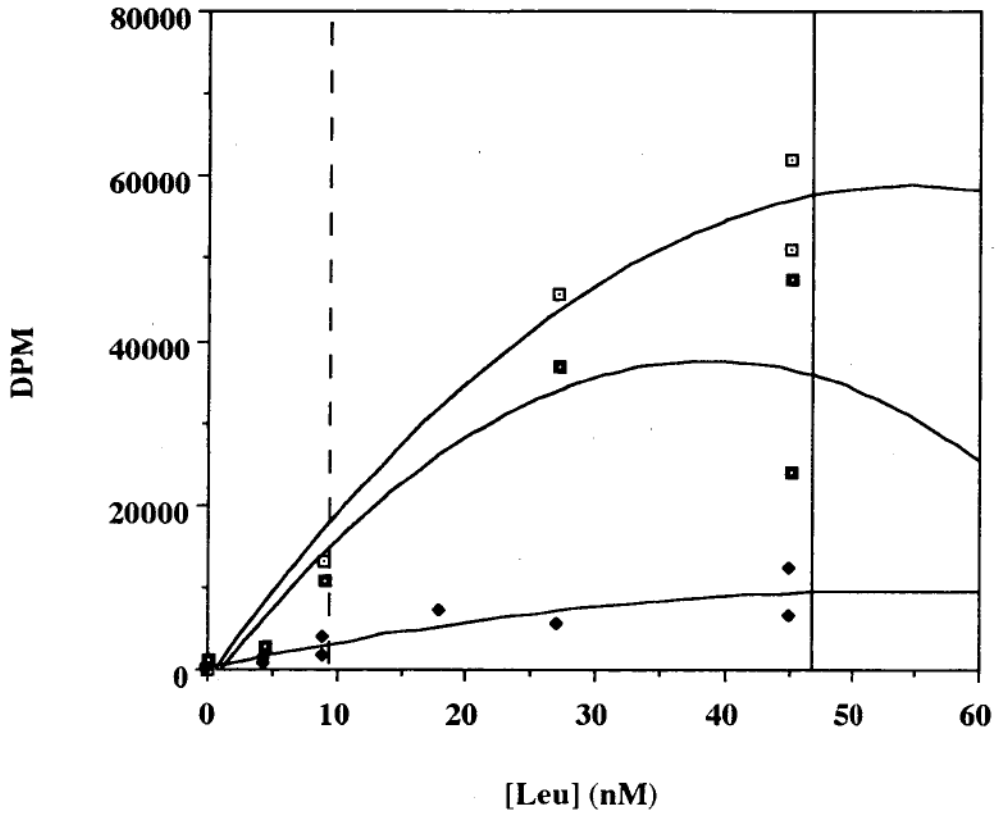


Figure 4.12: DPM versus Leu concentration for Organic Lake (3 m and 5 m depth) and the local seawater site (2 m depth) in June. The dotted line indicates the level of Leu used in this study. The solid line indicates approximate V_{max}.

(radioactive and non-radioactive) should pass through zero on the x and y axes. A regression which crosses the x-axis (substrate concentration) below zero indicates an exogenous pool of substrate equal to the absolute value of x when y equals zero (Moriarty, 1990). No clear trends emerged to suggest substantial use of exogenous leucine or thymidine by bacteria, so radioisotope incorporation values were not corrected for dilution of these isotopes. Insufficient data was taken to confidently determine the multiphasic nature of the isotope dilution plots. Figure 4.13 shows data from Organic Lake during January at 2 m depth (during peak productivity). Other plots are less clear (data shown in Appendix 7) but in this case (Figure 4.13) a third degree polynomial fitted better than a simple linear regression. There is no reason to believe that a polynomial should describe the data, other authors have plotted two straight lines or simply drawn curves through complex data sets (Pollard & Moriarty, 1984; Riemann *et al.*, 1982) or noted that data was highly variable and uninterpretable (García-Cantizano *et al.*, 1994; Bell, 1986).

To determine temperature optima of the bacterial consortia, radioisotope incorporation values were measured over a range of incubation temperatures. In all studies bacterial activity was shown to peak at about *in situ* temperature. In Ace Lake during July 1990 the bacterial populations below 6 m depth showed highest activity from 5 - 10 °C (Figure 4.14). In Ekho Lake, highest activity was at 15 °C and in Organic Lake highest activity was from 0 - 5 °C (Figure 4.15). Temperature optima were not determined in Fletcher Lake and the local sea water.

4.3.2 Routine radioisotope incorporation

4.3.2.1 Productivity experiments

Figure 4.16.1 shows the seasonal variation of bacterial production in Ekho Lake which provided a typical model for all the study sites. Figures 4.16.2, 4.16.3, 4.16.4 and 4.17 present Tdr incorporation rates for the other study lakes and the local seawater site. Each point is the average of 2-3 analysis replicates. In the Tdr incorporation experiments, the variation over season was consistent at all sites. The highest numbers of bacteria and highest concentrations of DOC occurred below the oxycline in Ekho Lake in August (with a monthly average of 2 hours sunlight per day), in Organic and Ace Lake in October and in Fletcher

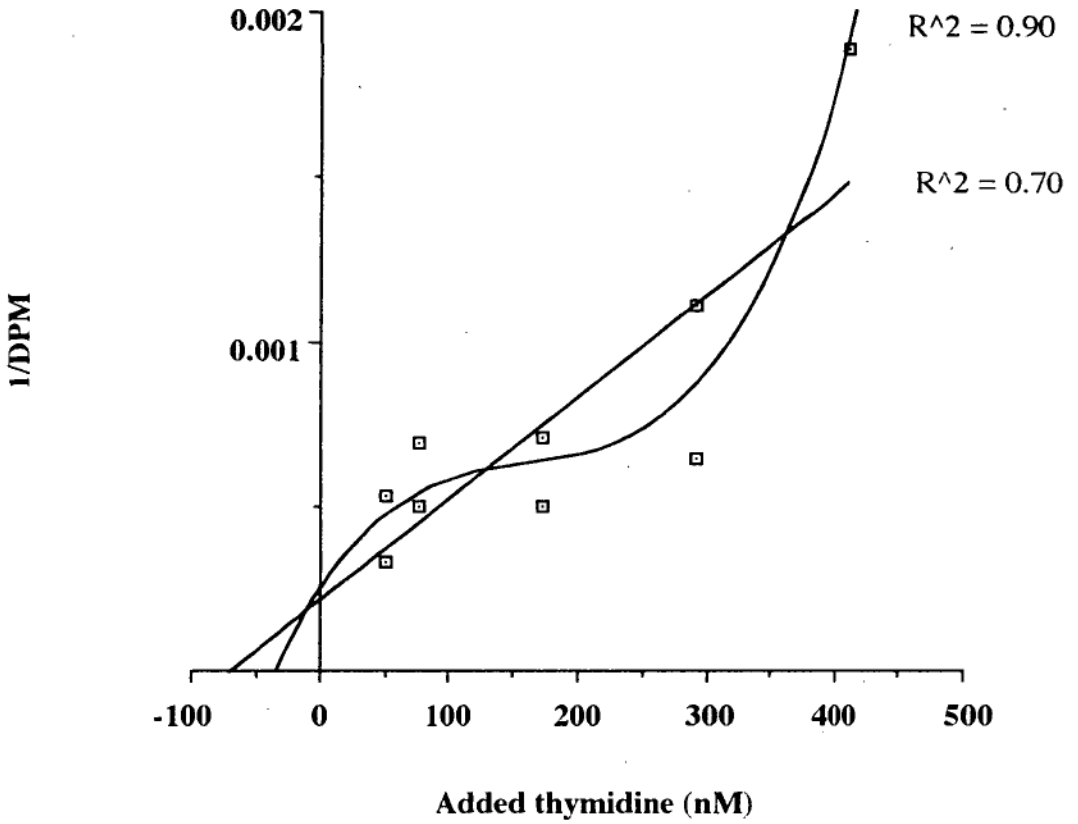


Figure 4.13: Isotope dilution plot for Tdr incorporation. Organic Lake 2 m depth, 8/1/1991. The lines crossing the x-axis below zero indicate an exogenous pool of thymidine.

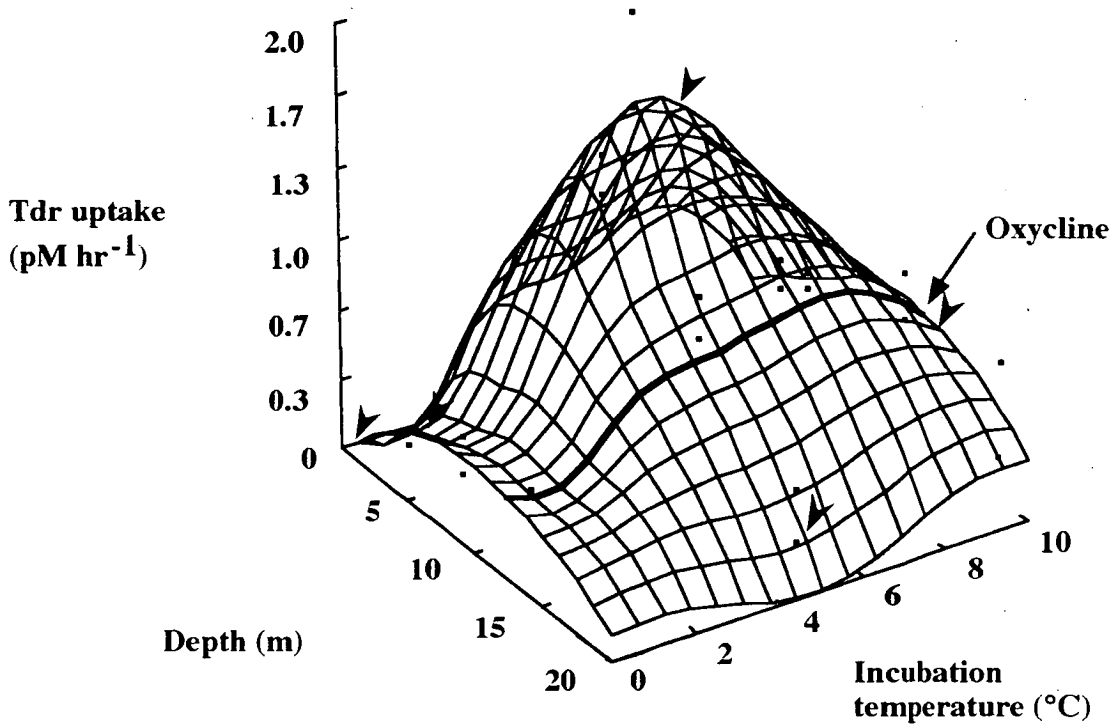


Figure 4.14: A surface plot (smoothed by weighted least squares method) of Tdr incorporation in Ace Lake (June 1990), versus depth and incubation temperature. *In situ* temperatures indicated by arrows. Below the oxycline Tdr incorporation may be underestimated.

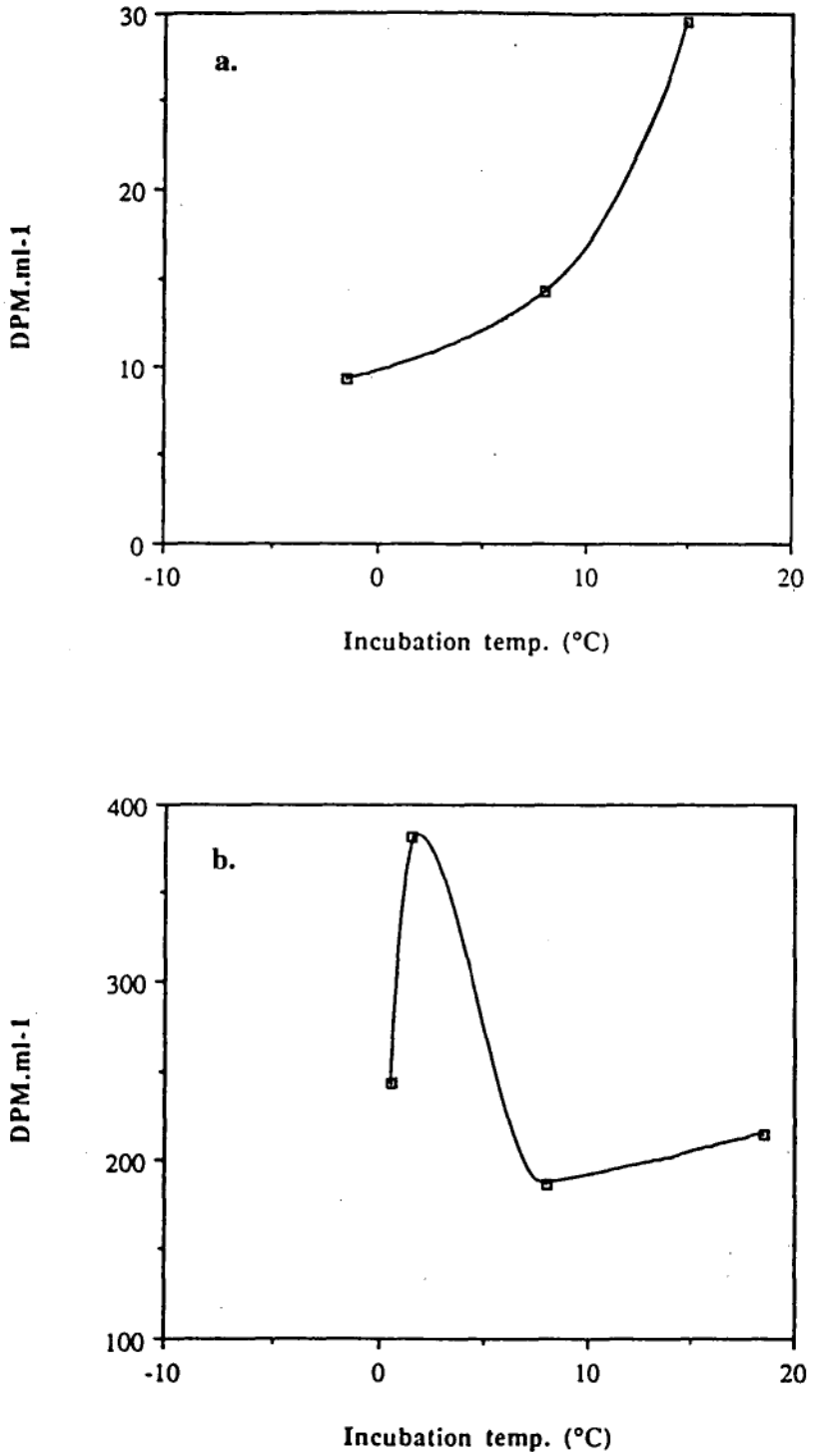


Figure 4.15: DPM versus incubation temperature for **a.** Ekho Lake at 6 m depth on the 18/5/1990. *In situ* temperature was 14.6 °C **b.** Organic Lake at 2 m depth on the 8/1/1991. *In situ* temperature was 5.9 °C.

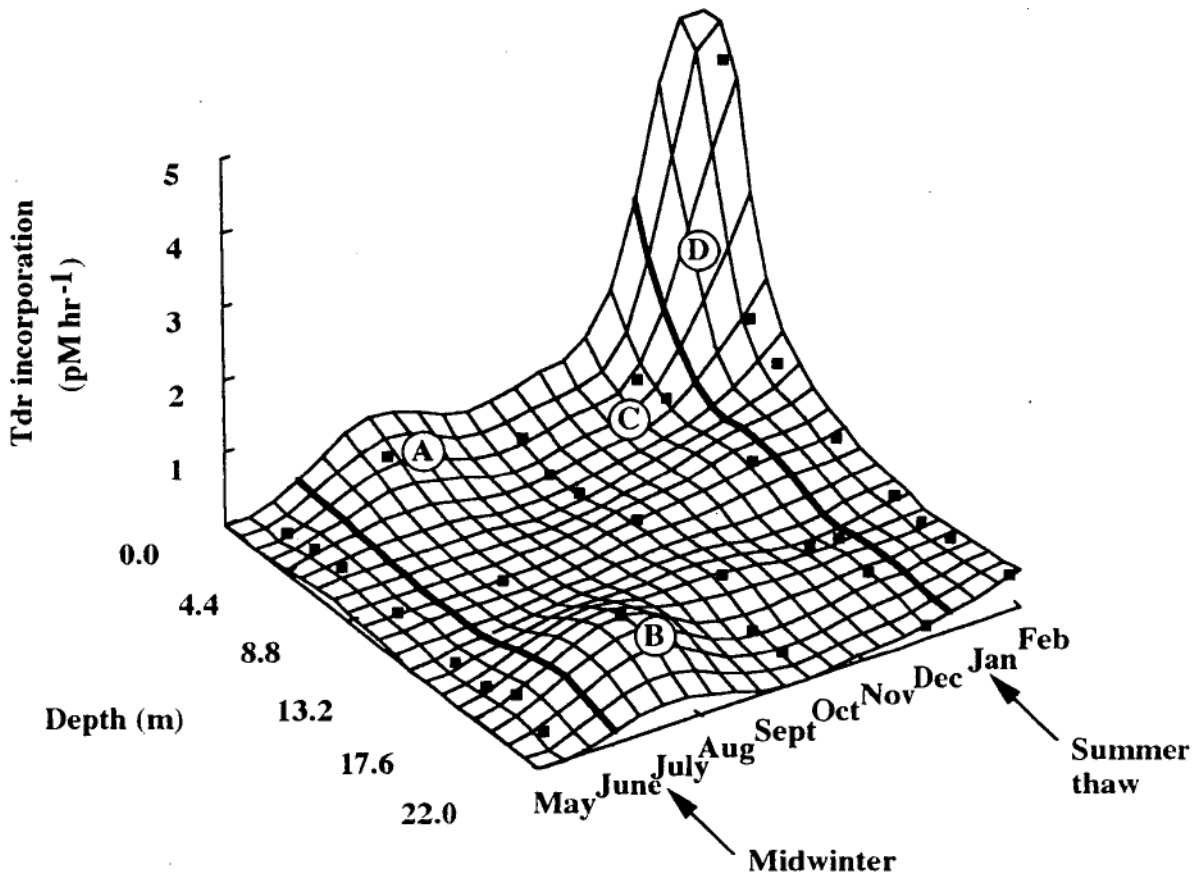


Figure 4.16.1: A surface plot (smoothed by the weighted least squares method) of Tdr incorporation in Ekho Lake against depth and date. Letters show significant peaks as explained in the text. Only data from the oxic waters has been shown as below the oxycline Tdr incorporation may be underestimated (Section 5.3.2.2).

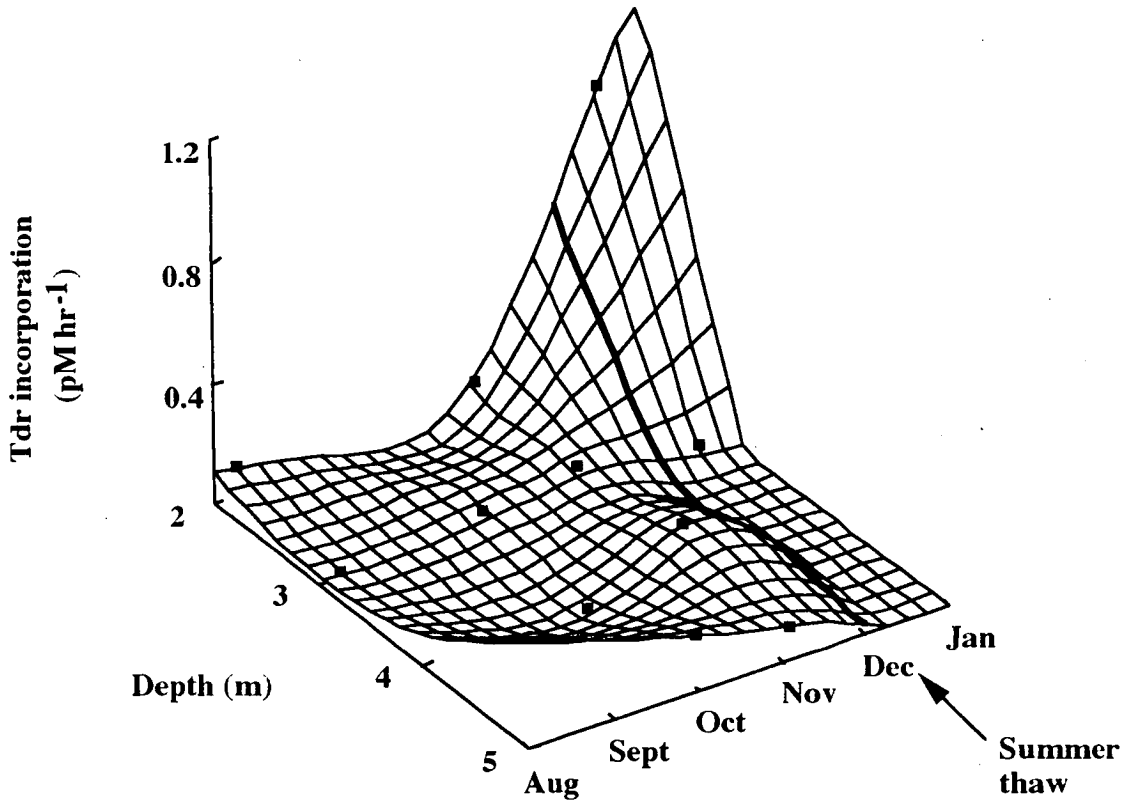


Figure 4.16.2: A surface plot (smoothed by the weighted least squares method) of Tdr incorporation in Organic Lake against depth and date. Below the oxycline Tdr incorporation may be underestimated (Section 5.3.2.2).

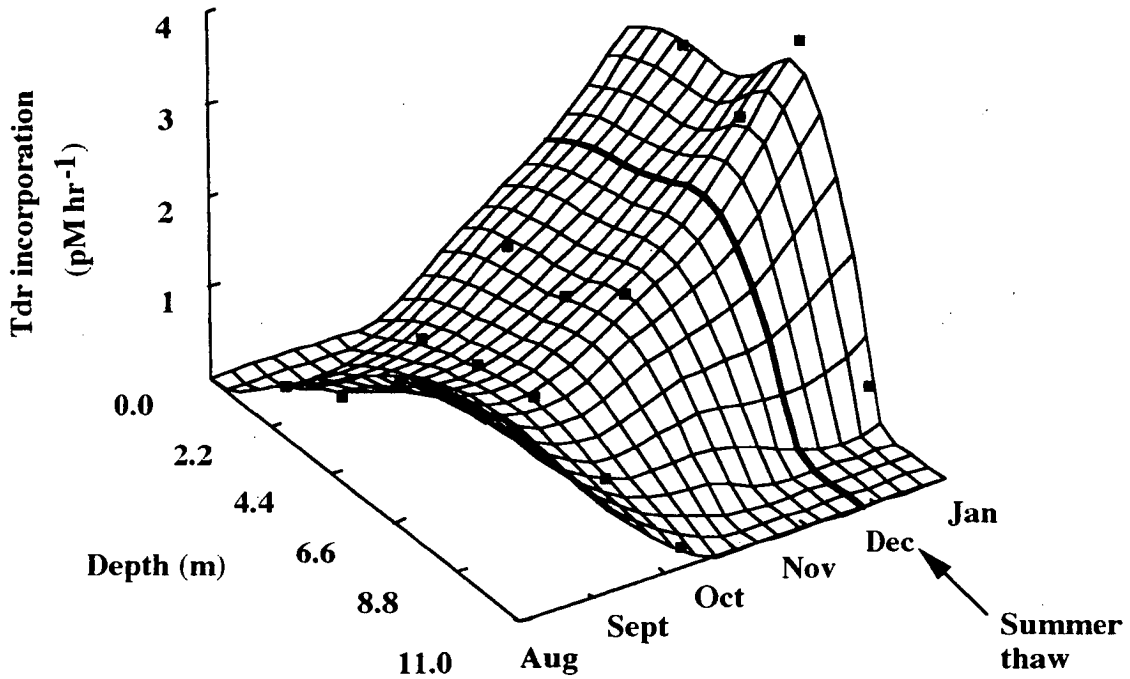


Figure 4.16.3: A surface plot (smoothed by the weighted least squares method) of Tdr incorporation in Fletcher Lake against depth and date. Only data from the oxic waters has been shown as below the oxycline Tdr incorporation may be underestimated (Section 5.3.2.2).

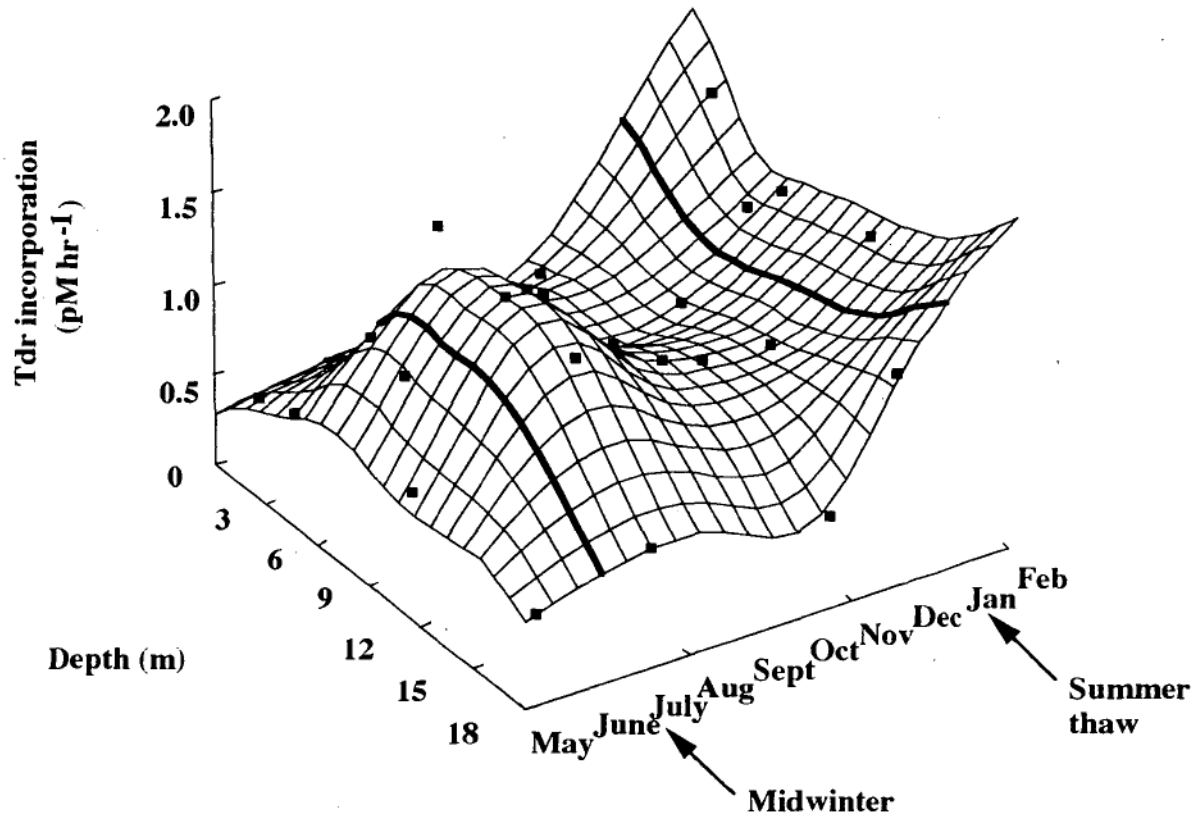


Figure 4.16.4: A surface plot (smoothed by the weighted least squares method) of Tdr incorporation in Ace Lake against depth and date. Only data from the oxic waters has been shown as below the oxycline Tdr incorporation may be underestimated (Section 5.3.2.2).

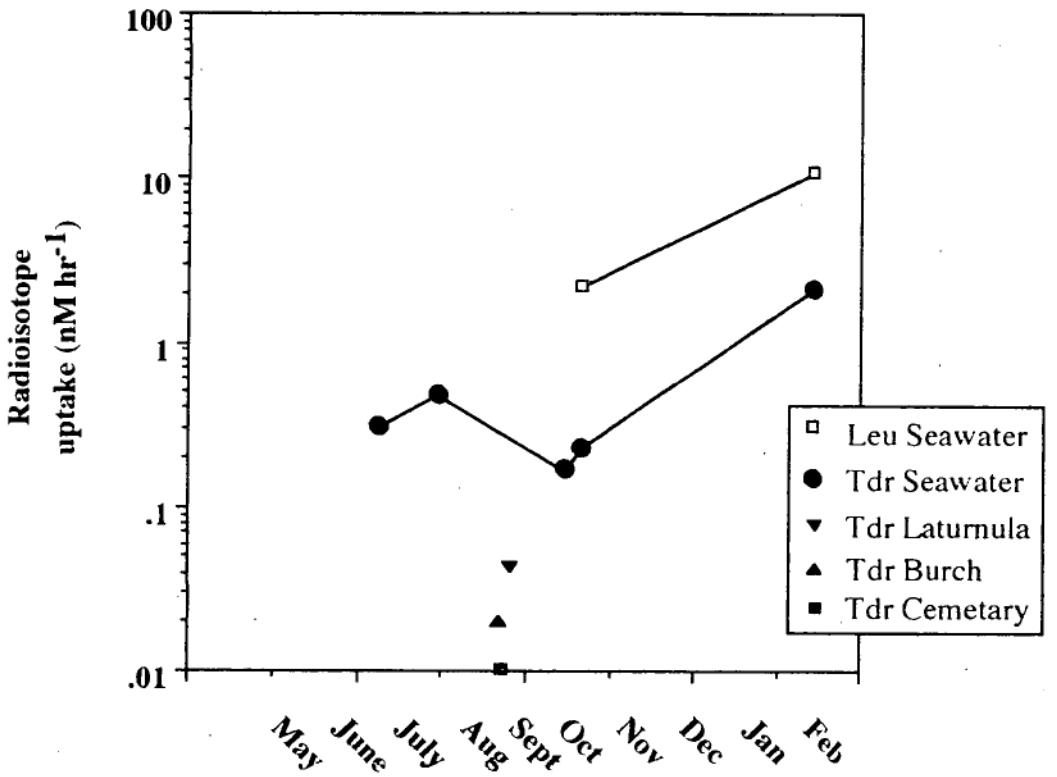


Figure 4.17: Radioisotope incorporation versus date for the local sea water (2 m depth), Burch Lake, Laturnula Lake and Cemetery Lake. Open symbols indicate Leu incorporation, closed symbols indicate Tdr incorporation.

Lake in November. Coincident with this increase in DOC concentrations and the general bacterial bloom, heterotrophic production, as determined by Tdr incorporation, increased just above the oxycline and to a lesser extent just below the ice cover (Figure 4.16.1; peaks A and B). At this time a peak in Tdr incorporation occurred in the local seawater, where chlorophyll concentration increased from 0.2 to 0.5 $\mu g\ l^{-1}$. During November 1990 (Figure 4.16.1; point C), when there was a monthly average of 11 hours sunlight per day, heterotrophic production increased in the oxylimnion at all sites. During January 1991, after the summer thaw when surface waters were most dilute (Figure 4.1.1), maximal productivity was recorded at a depth of 2 m at all sites (Figure 4.16.1; peak D) except Fletcher Lake where maximum productivity was at a depth of 6 m. The peak in Fletcher Lake corresponded with a dramatic increase in total bacterial numbers at the oxycline. After the summer thaw increased productivity occurred throughout the water column at all sites.

4.3.2.2 Protein synthesis experiments

Leu incorporation was only measured from August 1990 to January 1991. Unlike the Tdr data, the seasonal variation of Leu incorporation was not consistent throughout all sites (Figures 4.18.1, 4.18.2, 4.18.3, 4.18.4, 4.17). Leu incorporation in Organic Lake and Fletcher Lake peaked in November 1990 just above the oxycline at 94 and 57 pM leucine h^{-1} respectively. Leu incorporation in Ekho Lake peaked in January 1991 at 18 m depth at 23 pM leucine h^{-1}. There was a general increase in Leu incorporation in Ekho Lake at all depths during January 1991 and at 2 m depth in November 1990. Leu incorporation in Ace Lake peaked at 44 pM leucine h^{-1} in September 1990 just above the oxycline. Leu incorporation in the coastal marine site peaked at 11 pM leucine h^{-1} in January 1991. Leu incorporation did not appear to be strongly correlated to any other biological or physiochemical factor in the water column.

4.3.2.3 The ratio of [^{3}H]leucine incorporation to [methyl-^{3}H]thymidine incorporation

Table 4.2 shows generation times and protein synthesis rates for each site in early and late summer with accompanying site data. Tdr and Leu incorporation averages by month for all sites are given in Figure 4.19.

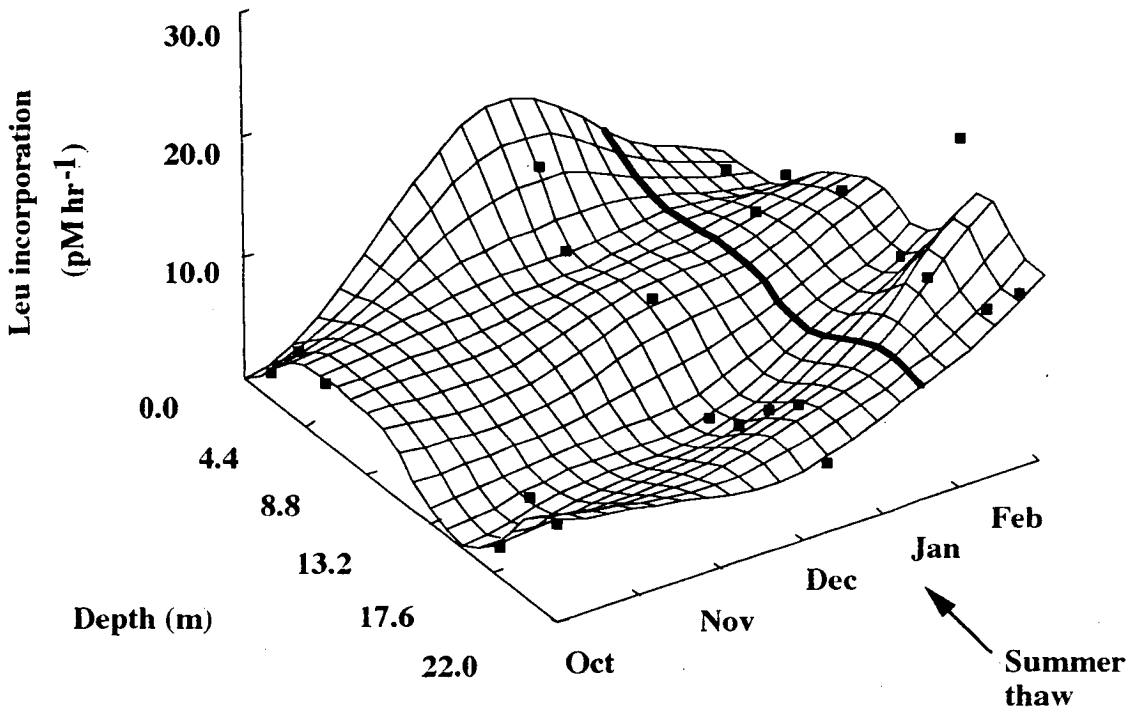


Figure 4.18.1: A surface plot (smoothed by the weighted least squares method) of Leu incorporation in Ekho Lake against depth and date.

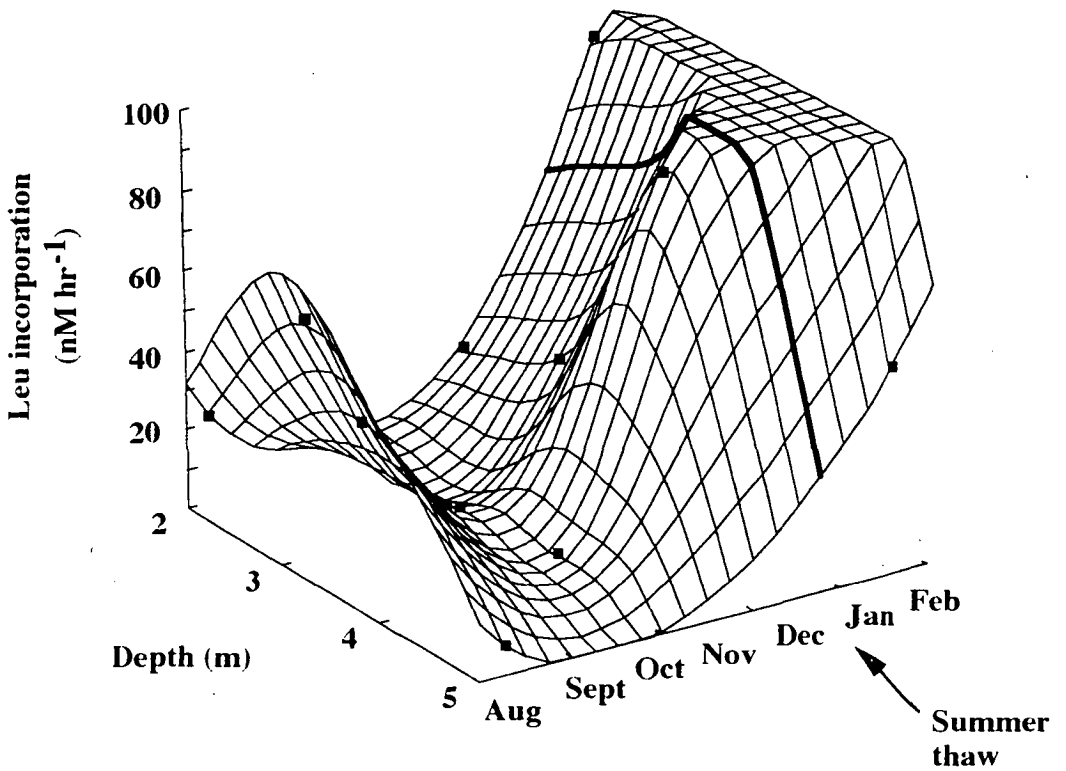


Figure 4.18.2: A surface plot (smoothed by the weighted least squares method) of Leu incorporation in Organic Lake against depth and date.

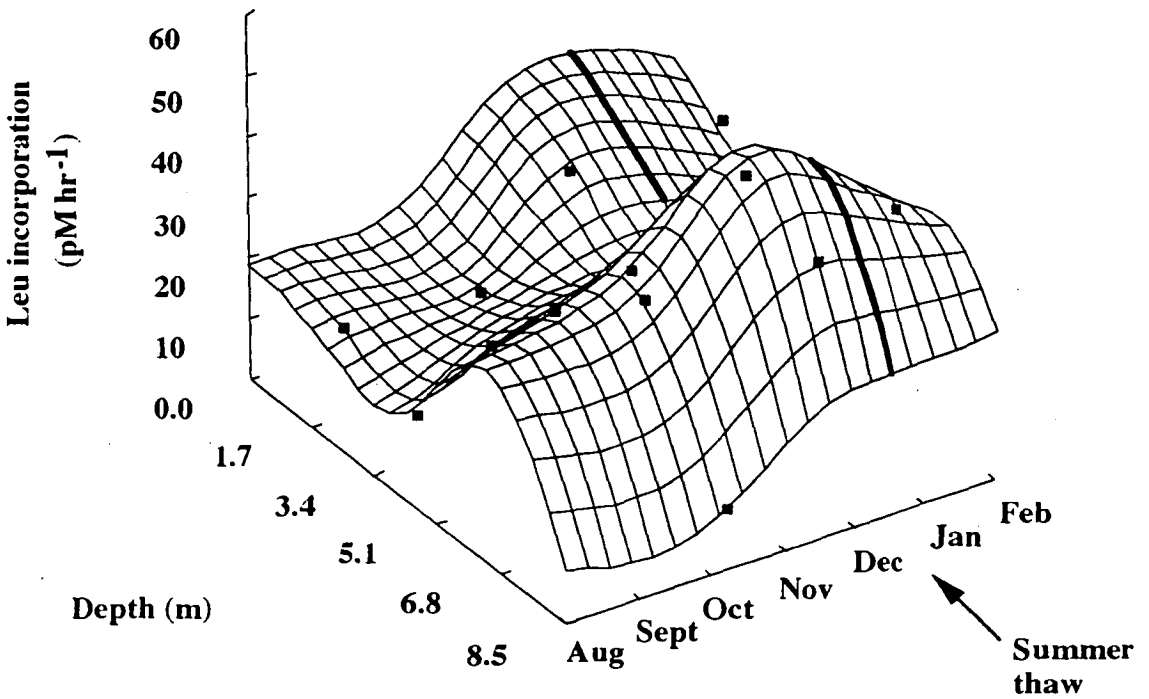


Figure 4.18.3: A surface plot (smoothed by the weighted least squares method) of Leu incorporation in Fletcher Lake against depth and date.

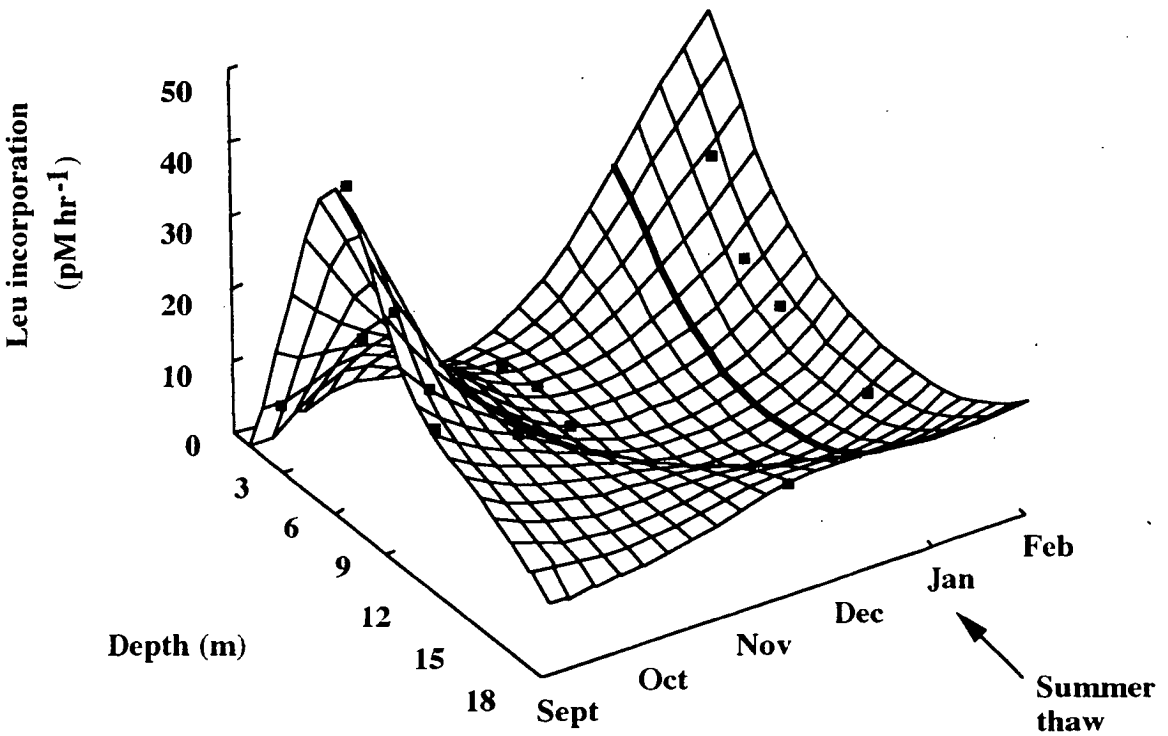


Figure 4.18.4: A surface plot (smoothed by the weighted least squares method) of Leu incorporation in Ace Lake against depth and date.

Table 4.2: Productivity characteristics of lake and seawater sites.

Site	Month (depth)	Generation (days)	Protein Syn. ($ng\ l^{-1}h^{-1}$)[c]	Ratio (Leu:Thy)[d]	Bacteria ($10^6\ ml^{-1}$)	DOC ($mg\ C\ l^{-1}$)	Salinity (‰)	Temperature (°C)
Organic Lake	January [a](2 m)	176	183	83	3.6	38.2	114	7.6
	August [b](3 m)	>364	118	2685	4.7	29.0	163	-6.3
Ekho Lake	January (2 m)	9	14	2	0.7	1.0	35	3.1
	August (18 m)	173	5	7	2.5	5.5	117	18.1
Fletcher Lake	January (2 m)	26	39	8	1.2	1.4	25	5.3
	August (6 m)	195	62	26	4.2	2.1	71	0.8
Ace Lake	January (2 m)	61	43	14	1.9	3.4	10	2.1
	August (6 m)	178	ND	ND	6.2	2.8	29	6.6
Sea Water	January (2 m)	36	20	5	1.3	0.3	34	-1.0
	August (2 m)	69	ND	ND	0.6	0.9	34	-1.8

[a] Samples taken between January 8th 1991 and January 31st 1991

[b] Samples taken between July 14th 1990 and August 16th 1990

[c] Conversion factor of Simon and Azam (1989) from Leu uptake

[d] Ratio of Leu uptake to Leu uptake

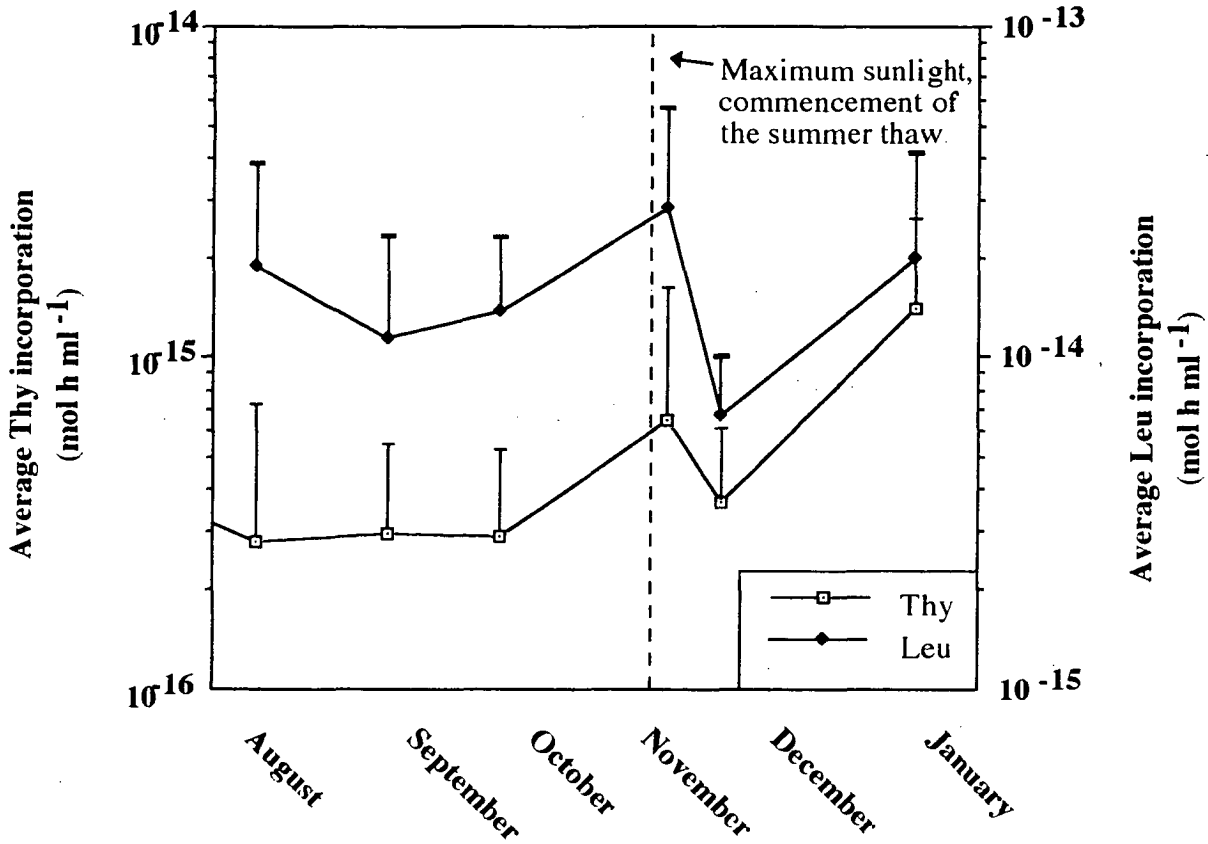


Figure 4.19: Average of Tdr and Leu incorporation by month for all sites. Median dashed line indicates the time of maximum sunlight and commencement of the summer thaw. Error bars are standard deviation, negative deviation has the same value as positive deviation but has been omitted for clarity.

Both Tdr and Leu incorporation increase at the time of maximum sunlight coincident with the commencement of the summer thaw. In mid-summer, after the thaw and when the mixolimnion was warmest and most dilute, another general increase was seen.

There was a general decrease in Tdr incorporation as salinity increased. Leu incorporation was highly variable but increased as salinity increased. Tdr and Leu incorporation for all sites plotted against salinity and temperature showed no easily elucidated trends (Figure 4.20.1, 4.20.2).

The ratio of Leu:Tdr for the whole data set was correlated (r^2=0.4, n=43) to DOC concentration and the log of generation time as determined by Tdr incorporation (r^2=0.4, n=48) but not to any other biological or physio-chemical factors in the water column. Replicate values for radioisotope incorporation data were averaged before the ratio was taken. When plotted against salinity and temperature (Figure 4.21), the ratio of Leu:Tdr corresponded with the theoretical model presented in Section 2.2.2 (Figure 2.2).

When *in situ* temperature was below -1.5 °C the sample was incubated at -1.5 °C. A linear model through ratio of Leu:Tdr data shows that it is reasonable to plot this data at -1.5 °C, the incubation temperature (Figure 4.22).

4.4 Dissolved organic carbon concentrations

This study confirmed expectations that DOC concentrations in the Vestfold Hills Lakes were strongly stratified, seasonally variable and associated with a number of environmental factors. Using the wet oxidation chemical/ultraviolet methods developed by Hine and Bursill (1985) sensitivity down to 0.1 mg C l^{-1} was achieved. This allowed quantification of local seawater (0.3 - 1.6 mg C l^{-1}) through to anaerobic waters at the bottom of Organic Lake (up to 74 mg C l^{-1}). Fractionation using filters showed rapid utilisation of <0.2 μm DOC produced by photosynthesising bacteria in the anaerobic lake waters as light levels increased during early summer (Figure 4.23.1, 4.23.2). As mentioned in the previous section, when plotted against salinity and temperature, DOC correlated strongly with the 'harshness' (as indicated by elevated

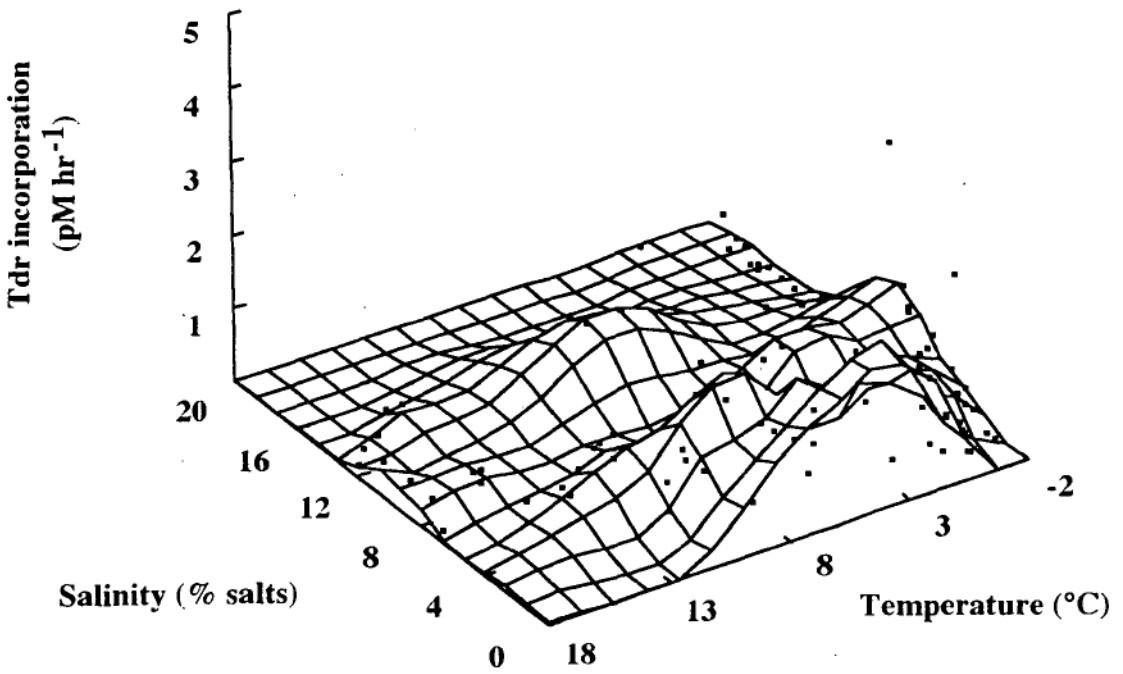


Figure 4.20.1: A surface plot (smoothed by the weighted least squares method) of Tdr incorporation in all sites against salinity and temperature.

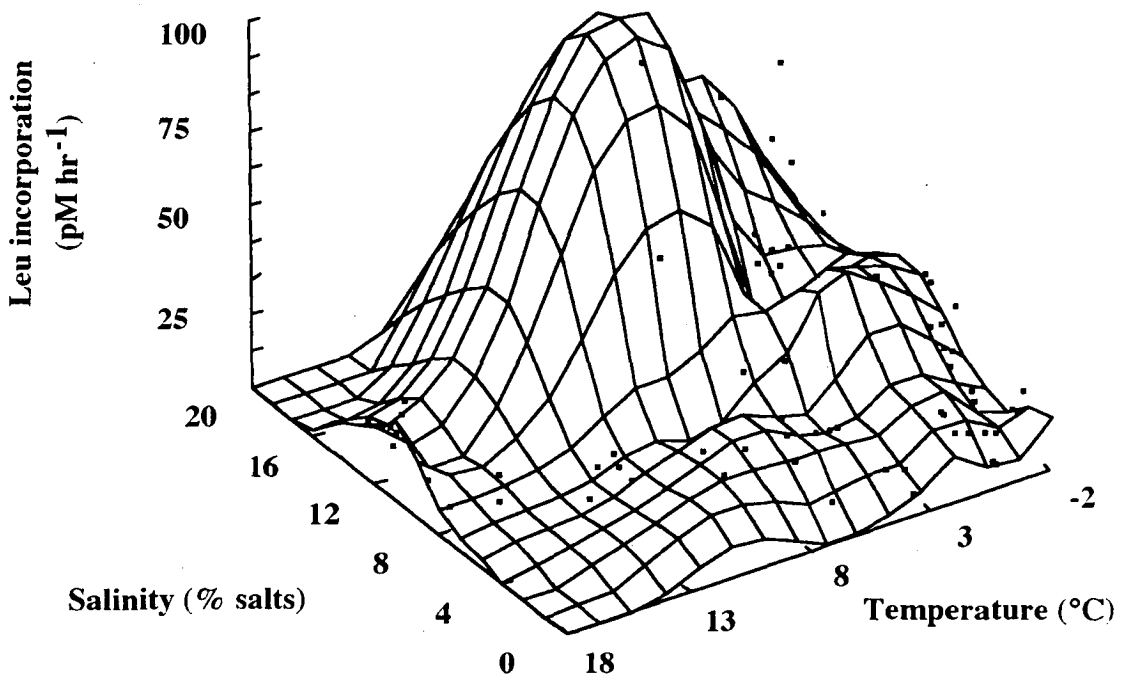


Figure 4.20.2: A surface plot (smoothed by the weighted least squares method) of Leu incorporation in all sites against salinity and temperature.

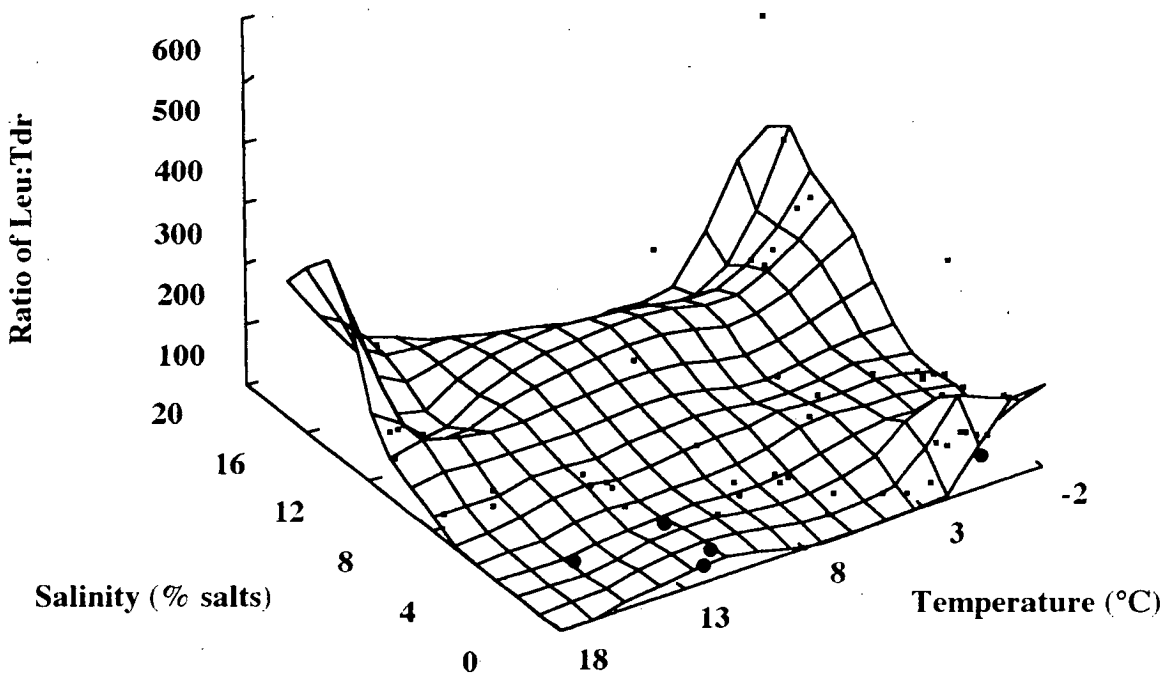


Figure 4.21: A surface plot (smoothed by weighted least squares method) of the ratio of Leu:Tdr versus salinity and temperature. Large closed circles are data from other studies (Grossmann & Diekmann, 1994; Kirchman, 1990; Riemann & Bell, 1990; Servais & Garnier, 1993; Simon & Azam, 1989).

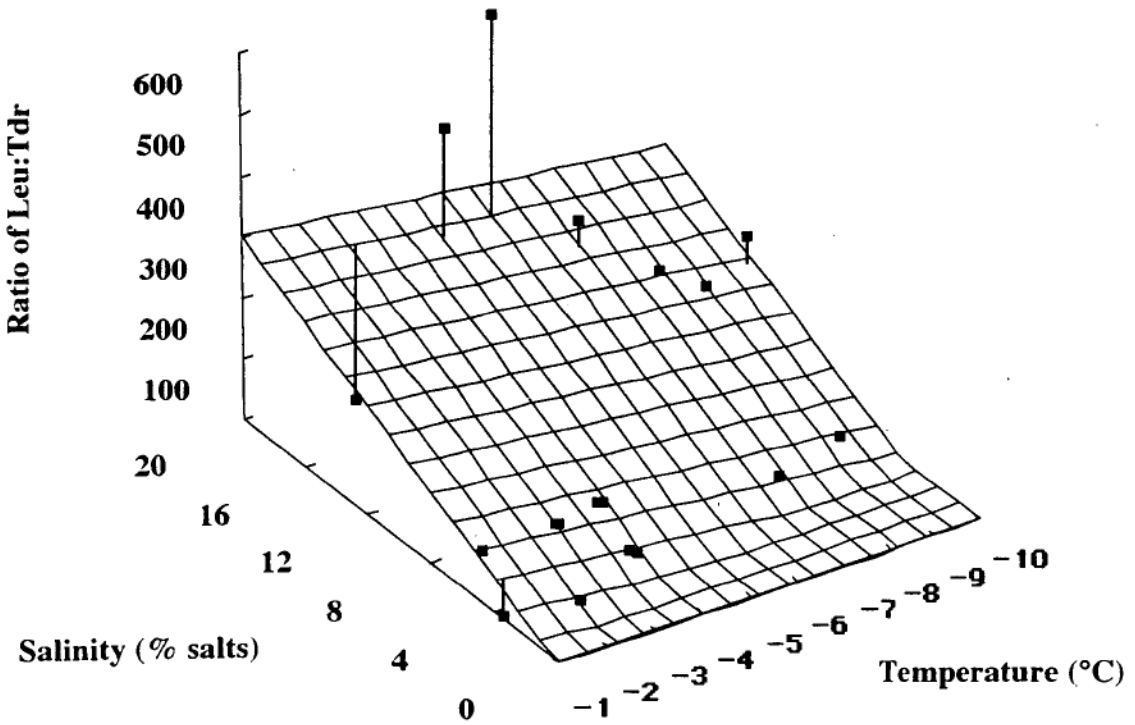


Figure 4.22: A linear regression surface plot of the ratio of Leu:Tdr determined at -1.5 °C versus salinity and *in situ* temperature. Data was from all sample sites with *in situ* temperatures ≤ -1.5 °C.

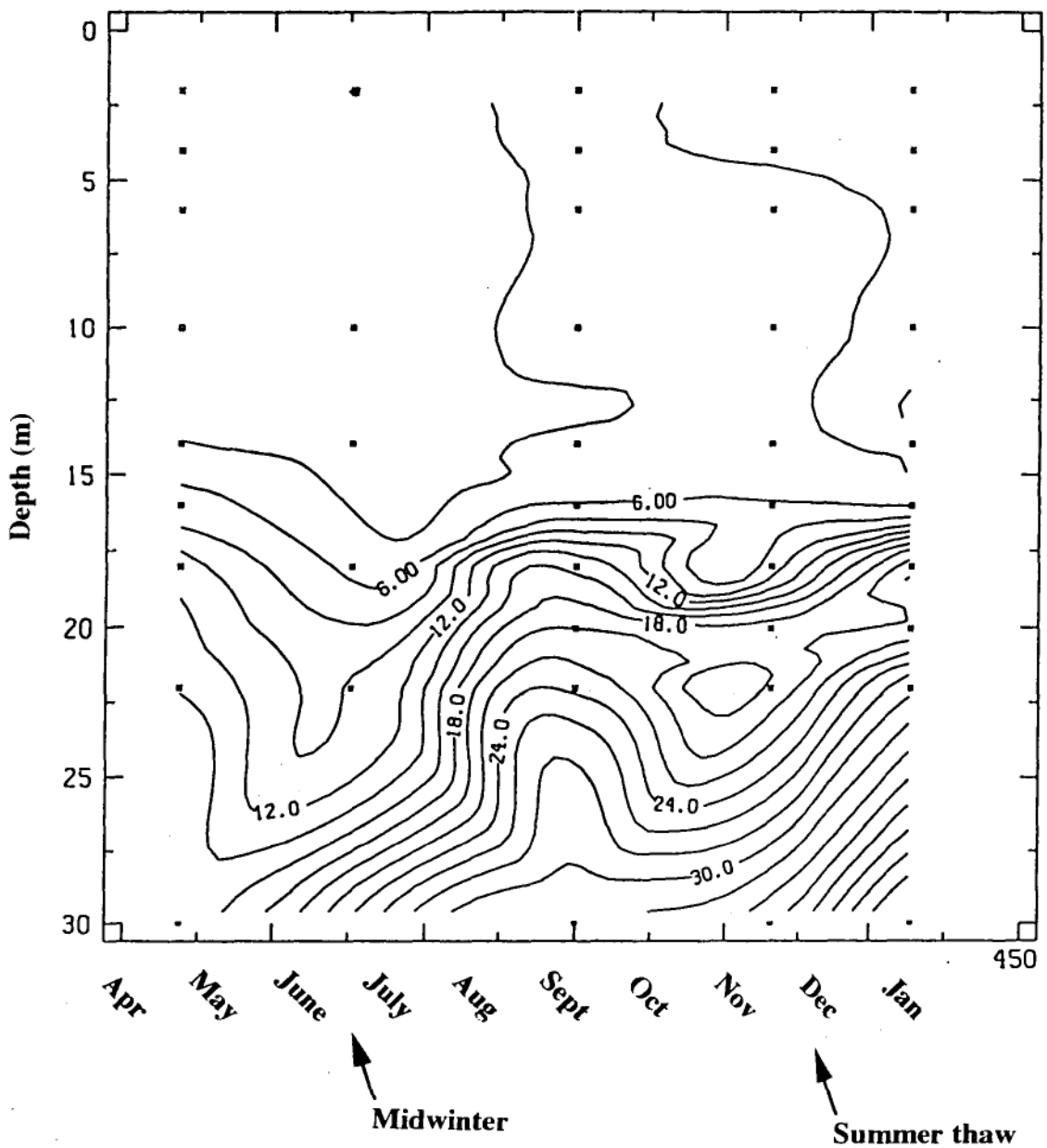


Figure 4.23.1: Isopleths of total DOC fraction (mg l^{-1}) for Ekho Lake versus depth and date.

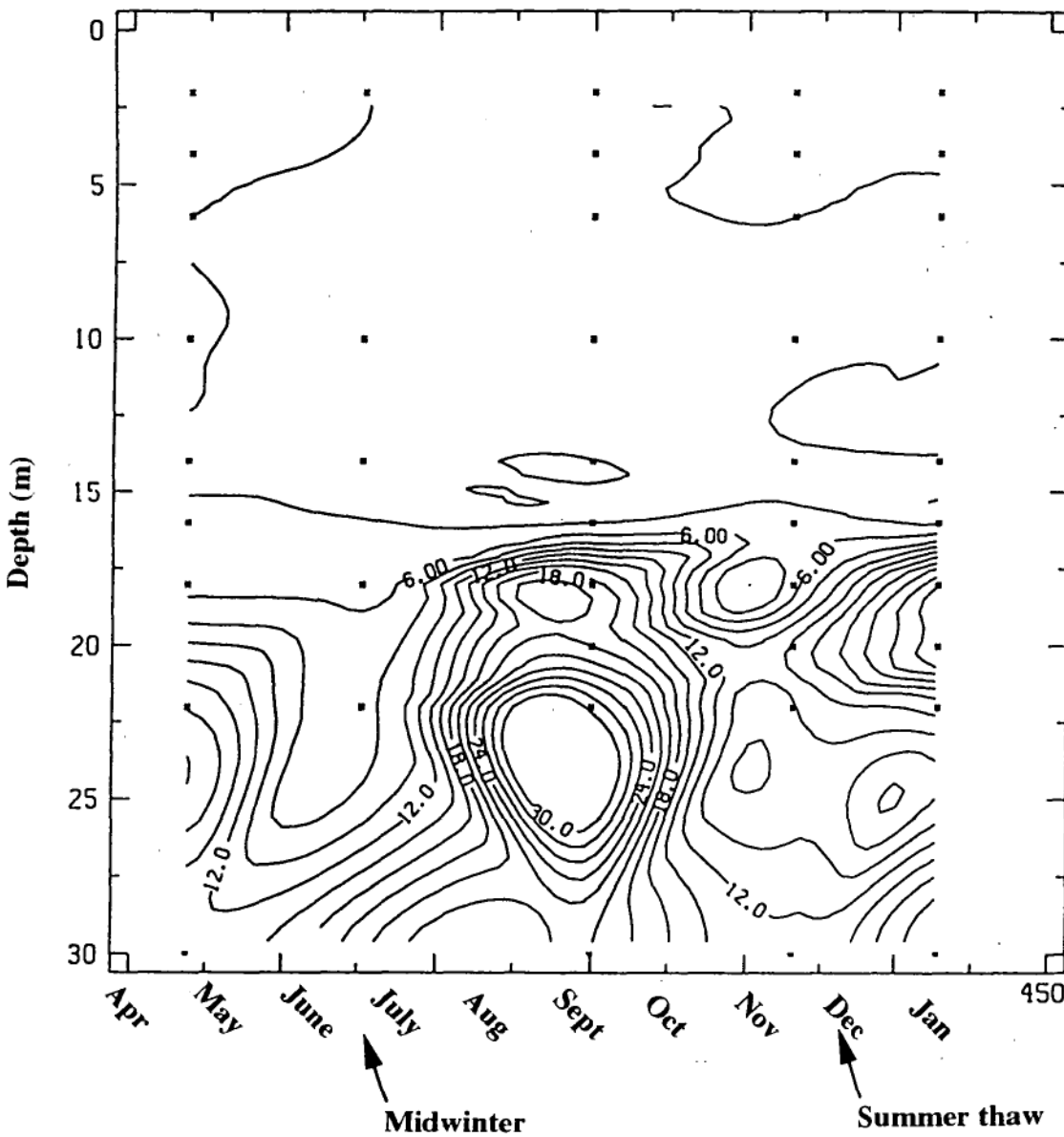


Figure 4.23.2: Isopleths of < 0.2 μm DOC fraction (mg l^{-1}) for Ekho Lake versus depth and date.

ratio of Leu:Tdr) of the lakes, showing that DOC accumulated in waters where salinity or temperature slowed bacterial activity.

DOC in the oxylimnion of each lake was between 2 and 6 mg carbon per litre (mg C l^{-1}), decreasing slightly throughout the year; except in Organic Lake, the coldest, most saline of the study sites, where the upper waters had 23 to 38 mg C l^{-1}. In all lakes, DOC concentrations were 2 to 10 times greater in the oxycline and anoxylimnion than in the oxylimnion. Local seawater had between 0.3 and 1.6 mg C l^{-1} DOC. 0.2 μm filtered DOC concentrations were significantly correlated (r=0.6, n=97) to pH.

A general increase of DOC in anaerobic lake waters was associated with a photosynthetic bacterial bloom and occurred below the oxycline in Ekho Lake during August (with a monthly average of 2 hours sunlight per day), in Organic and Ace Lake in October and in Fletcher Lake in November. DOC peaks in anaerobic waters were associated with smaller peaks in aerobic waters. Though it was not investigated in this study, the smaller peaks could have been from increased phytoplankton activity. During September DOC concentration increased in the local seawater, associated with an increase in chlorophyll concentration.

Changes in DOC concentrations were not associated with changes in species composition but were closely associated with peaks in total bacteria count at the oxycline and in the anoxylimnion. As low levels of bacterial activity were found by radioisotope incorporation methods to occur at and below 1 °C the storage of DOC samples in future studies should be for as short a time as possible or at lower temperature.

4.4.1 Filtered organic carbon concentrations

Filtered organic carbon values were generally higher than values obtained from the measurement of unfiltered samples (Figure 4.24). A possible reason for this was the harshness of the filtering process. It was likely that some floccules and more fragile cells may have broken up releasing more organic carbon into the sample. Most of the organic carbon measured passes through a 1.0 μm filter. Though the overall trend within the filtered fractions follows the unfiltered organic carbon, individual features presented themselves.

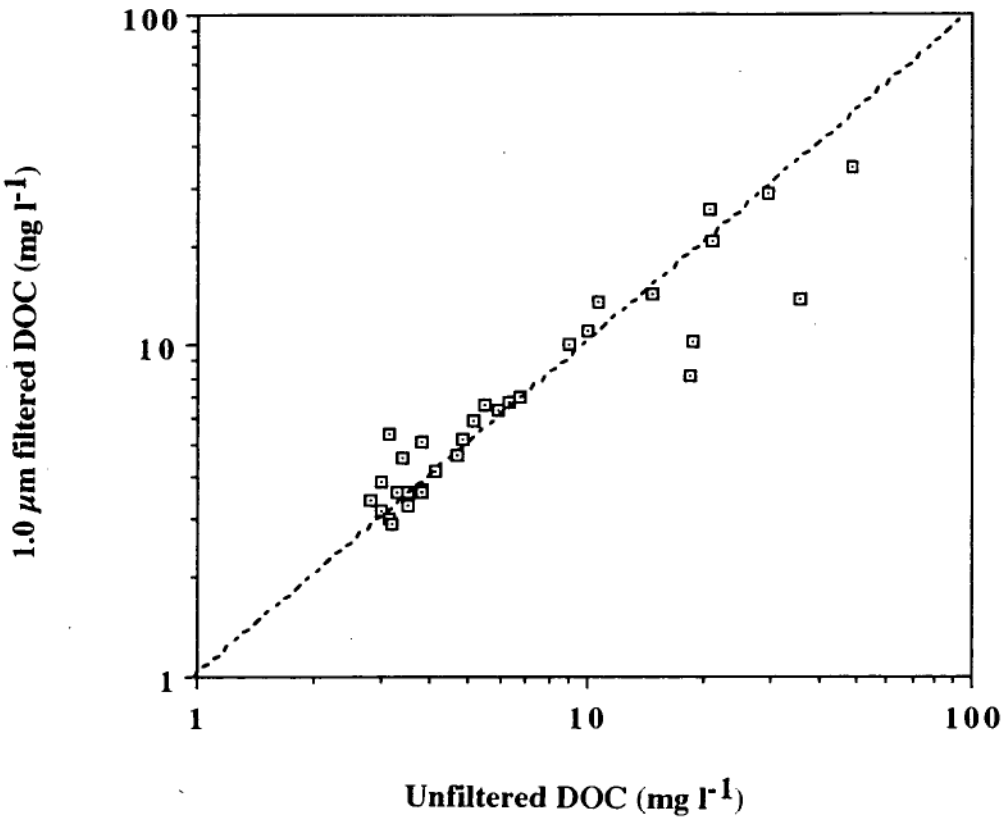


Figure 4.24: Ekho Lake 1.0 μm filtered DOC fraction versus organic carbon measurement obtained from unfiltered samples. Data is from all sample sites. Dotted line follows equal values.

In Organic Lake all fractions peaked in September at 5 m except the <0.2 μm fraction which peaked at 4 m. In Ekho Lake, all fractions peaked in January at 20 to 30 m. The <0.2 μm fraction also peaked in September at 25 m. In Fletcher Lake the <1.0 and <0.4 μm fractions peaked in October at 9 m. The <0.2 μm fraction peaked in November at 12 m and was generally low. Ace lake, the least saline of the sites sampled had low DOC concentrations, the anoxylimnion had DOC concentrations around 10 to 15 mg C l^{-1}. Unlike the other lakes, the fractions did not require dilution before analysis. All fractions peaked in May at 18 m, the <1.0 and <0.6 μm fractions also peaked in October around 16 m. There was a general correlation for the whole data set (all lakes and seawater site) between total bacterial numbers and <0.2 μm filtered DOC fraction (r^2 = 0.4). Figure 4.25 shows total bacterial count versus the <0.2 μm DOC fraction for all Ekho Lake samples.

4.5 Measurement of other environmental variables

Monthly meteorological reports provided an indication of the weather at Davis Station over the year, a graphical summary is presented in Figure 4.26.

4.5.1 Temperature

Temperatures were measured to ±0.1 °C using both the DMA 35 and Kammerer bottle thermometer. The temperature of the top 3 to 6 m of the lakes varied with ice or surface temperature. In Ace Lake and Ekho Lake the haloclines were abrupt enough to form a distinct layer heated by the sun (Heliothermal layer; Figure 4.27). If the lake was sufficiently deep, the anoxylimnion was thermally stable. Figure 4.27 also illustrates that, due to the latent heat of ice, between June and December the heat from the sun is adsorbed by the ice with no corresponding rise in temperature. Data for Ekho Lake is presented in Figure 4.28. Other temperature data is detailed in Appendix 7.

4.5.2 Density

Densities taken using the hand refractometer had an accuracy of ± 0.002 g ml^{-1} though mixing of sample as the Kammerer bottle rose through the water column affected the accuracy of these results by up to 0.01 g ml^{-1} (Figure 3.1). Densities were presented as salinity (‰) for ease of

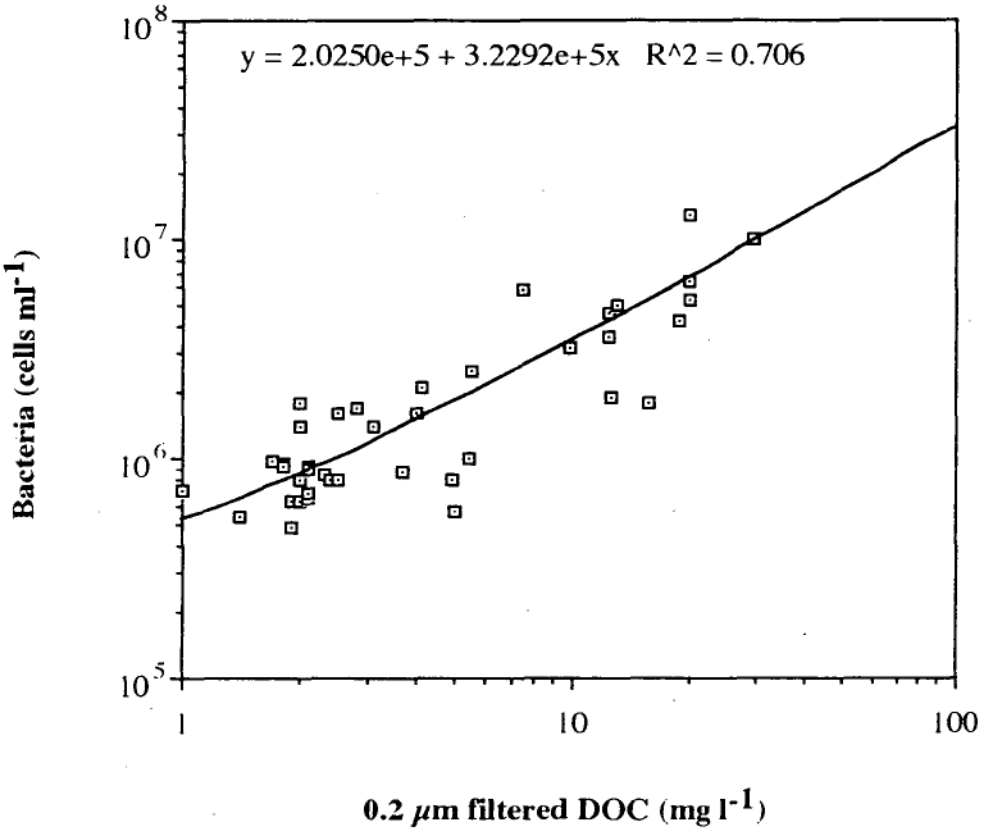


Figure 4.25: Total bacterial count versus the <0.2 μm DOC fraction for all samples from Ekho Lake.

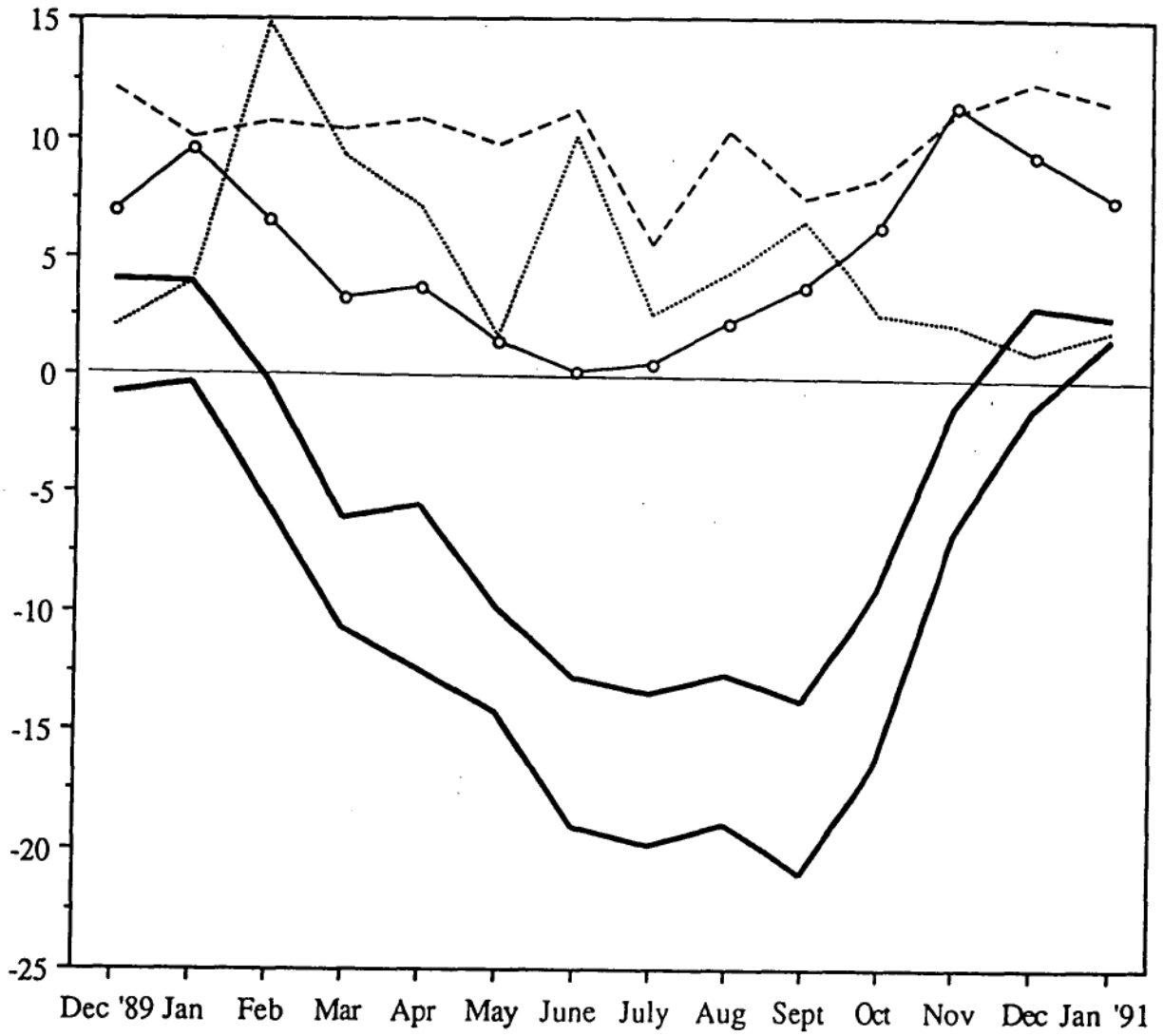


Figure 4.26: A graphical summary of weather conditions at Davis station during 1990. Averages by month as taken from the monthly meteorological report for; av. min. temperature (°C; ——), av. max. temperature (°C; ——), average hours sunshine per day (—o—), average wind speed (kts; - - - -) and total snowfall (mm; ·····).

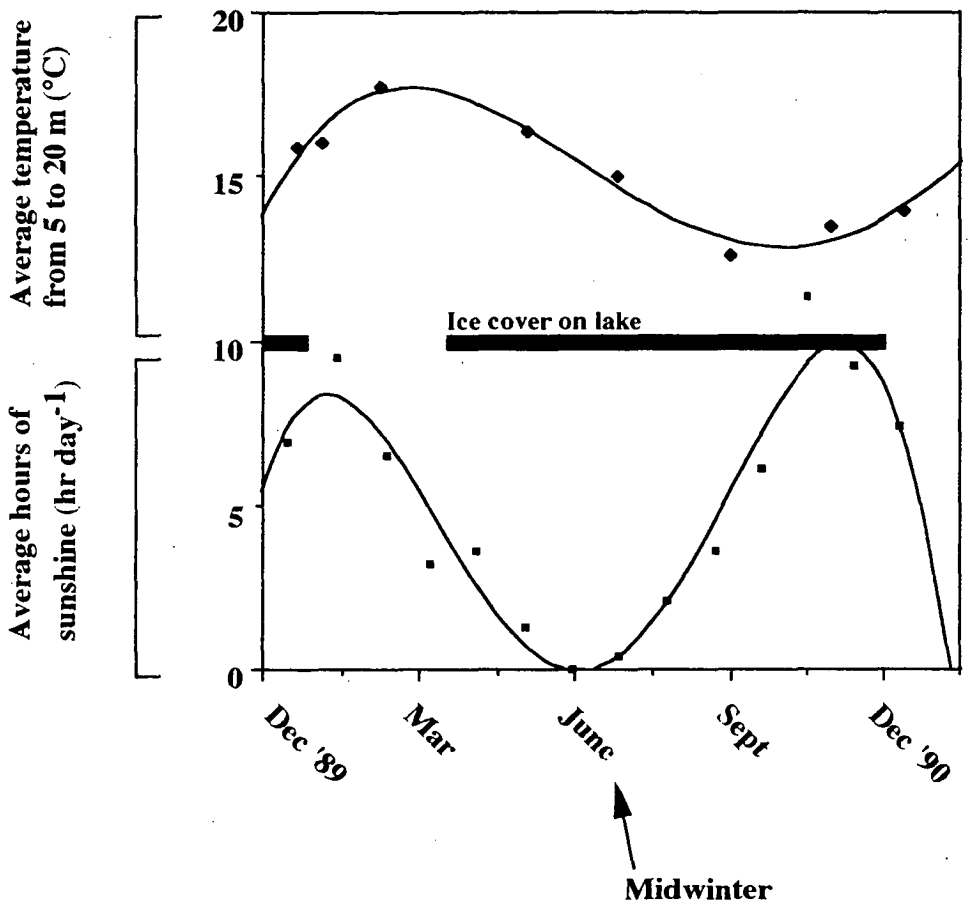


Figure 4.27: Average temperature from 5 to 20 m depth for Ekho Lake and average hours of sunshine per day versus date. Median solid line indicates duration of lake ice cover.

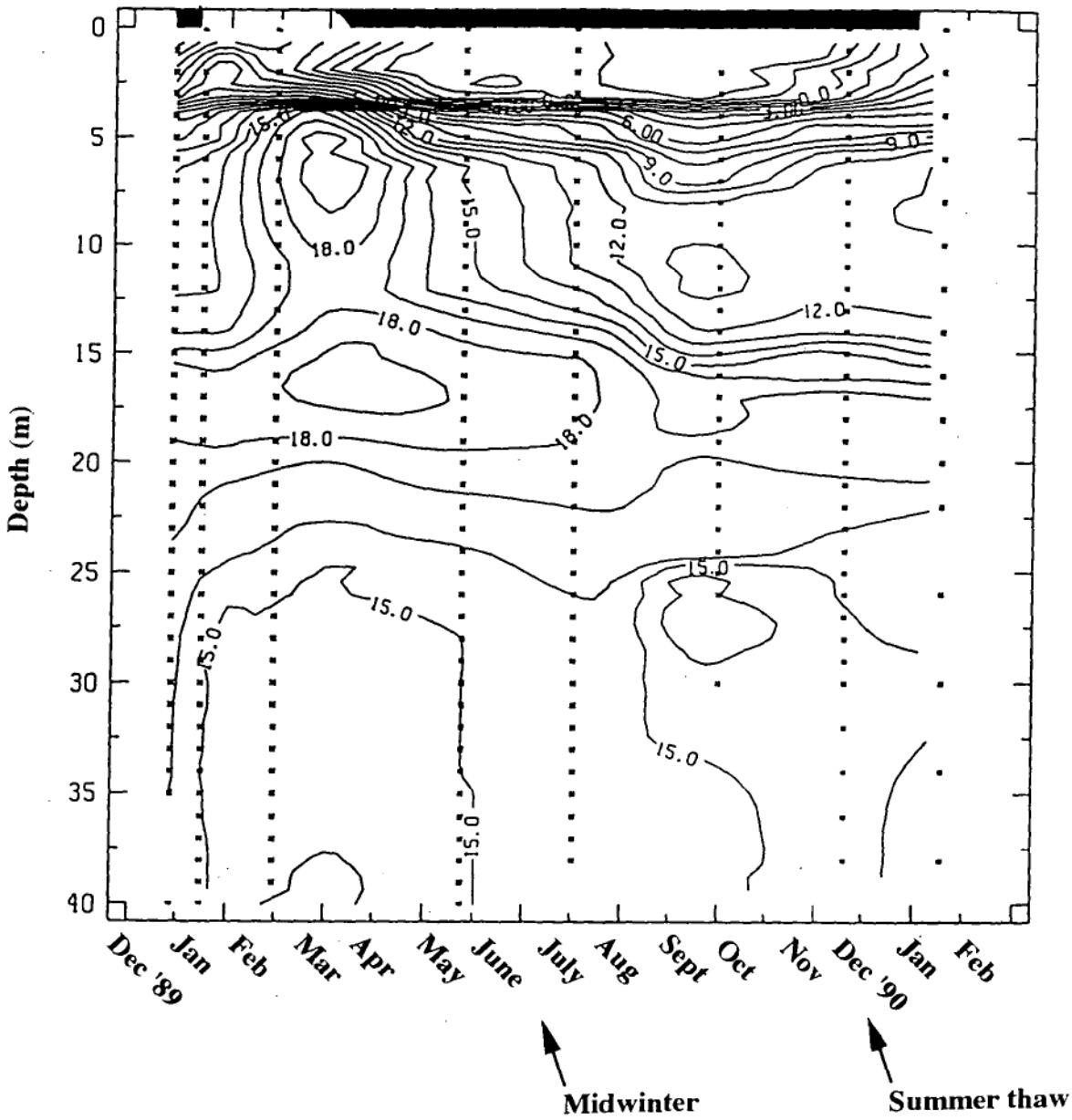


Figure 4.28: Isopleths of temperature (°C) in Ekho Lake versus depth and date. The solid line at 0 m depth indicates duration of lake ice cover. Data points are indicated by asterisks.

interpretation. Lakes mixed to between 3 and 5 m and slowly increased in salinity to the oxycline. The anoxylimnion was more dense and fairly stable. Data for Ekho Lake is presented in Figure 4.29. Other density data is presented in Appendix 7.

4.5.3 Oxygen

In each of the lakes the oxycline remained relatively stable during the year. It was indicated by a visible layering within the Kammerer bottle sampler and a definite smell of H_2S. This layer was present at; 4.5 m in Organic Lake, 22 m in Ekho Lake, 8.5 m in Fletcher Lake and around 11 m in Ace Lake. The Winkler method indicated that oxygen was still present at and about those levels. However, the anoxylimnion contains sulphides, iron, organics and nitrates which can affect the accuracy of the Winkler method (Strickland & Parsons, 1972). Due to the extreme variance in the compositions of the lakes, the effects were not determined. The Winkler determinations below the oxycline were considered unreliable and were ignored. The oxycline and anoxylimnion in all lakes was determined by the smell and physical appearance of the sample and the reaction to resazurin (a reduction indicator).

In the oxylimnion, oxygen concentration dropped during the middle of winter, increased rapidly with increasing light to peak in late November in the upper waters and decreased again during January. Oxygen concentrations in Organic Lake had a peak value of 15 ml l^{-1} at 3 m during November. This concentration seemed quite high compared to routine values but in both Fletcher Lake and Ace Lake the oxygen concentrations peaked at about the same time and depth. Oxygen maxima coincide with the maximum average hours of sunshine per day. Fletcher Lake mixed to 6 m in January, with very high heterotrophic productivity peaking at 6 m. Coincidentally oxygen concentrations were lowest at 6 m (2.1 ml l^{-1}). Oxygen concentration data is presented in Appendix 7.

4.5.4 pH

All sites had circumneutral pH values of 7.0 - 8.6. pH was fairly constant at 8 to 8.5 in the upper waters and about 7.5 below the oxycline in all lakes except Organic Lake where the pH was between 7.0 and 7.4.

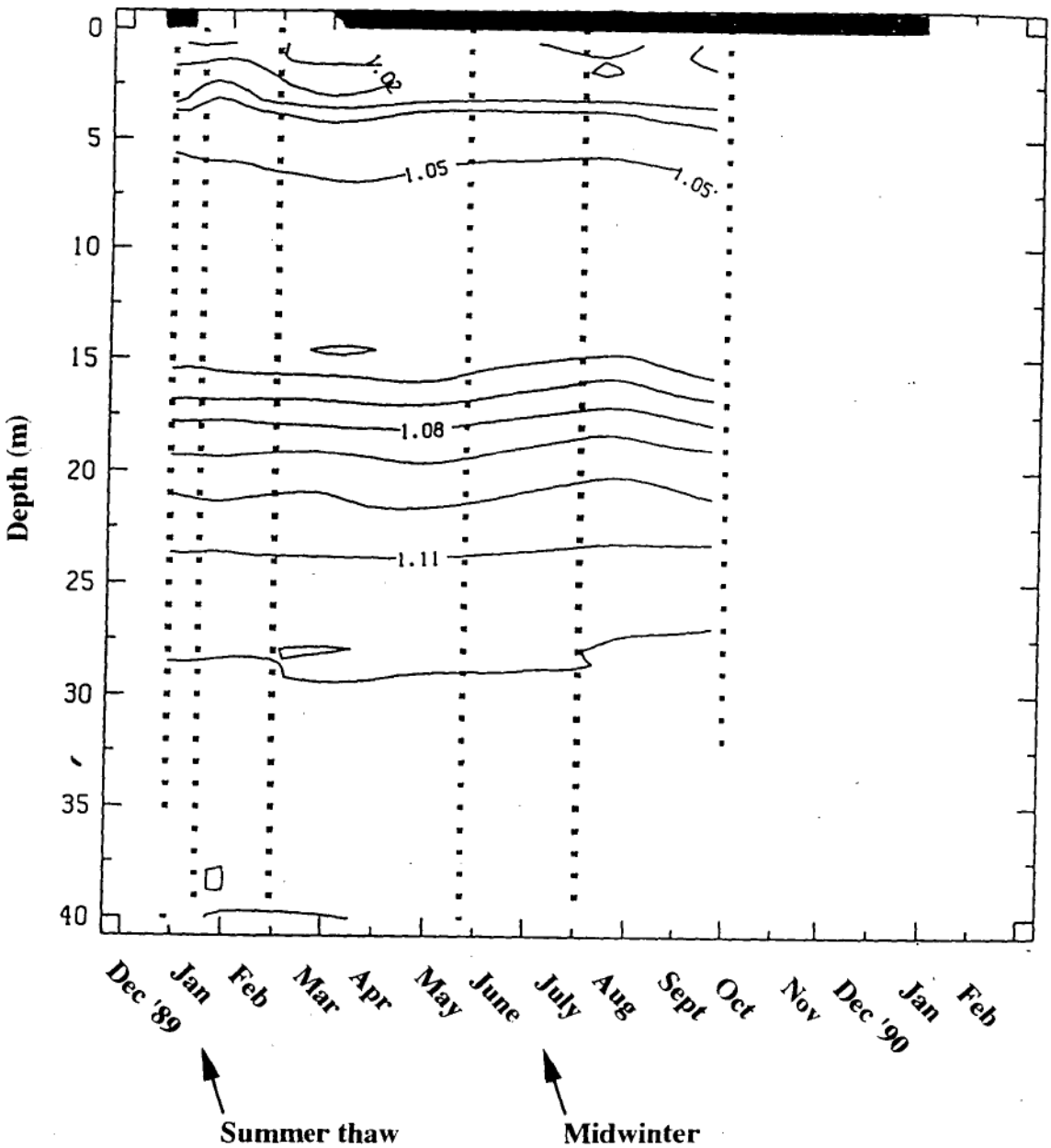


Figure 4.29: Isopleths of salinity (g ml^{-1}) in Ekho Lake versus depth and date. The solid line at 0 m depth indicates duration of lake ice cover. Data points are indicated by asterisks.

correlated well with 0.2μm filtered DOC concentrations (r^2 = 0.6) and salt concentration (r^2 = 0.4). pH data is presented in Appendix 7.

4.5.5 Chlorophyll

Chlorophyll concentrations in the oxylimnion were generally low. Chlorophyll concentrations peaked in Ace Lake and Fletcher Lake during November just below the oxycline. At this time Fletcher Lake water was bright emerald green (with 188 μg chlorophyll l^{-1}). This was the highest concentration of chlorophyll of all the samples analysed. Microscopy of the sample did not show high concentrations of algae. In Ace Lake, chlorophyll concentration peaked in November at 11 m (27 μg l^{-1}) and also in May at the same depth (18 μg l^{-1}). In Ekho Lake chlorophyll concentration peaked in May at 16 m (39 μg l^{-1}). Chlorophyll concentrations in the oxylimnion generally remained below 1 μg l^{-1}. Chlorophyll concentration data is presented in Appendix 7.

4.6 Bacterial reduction of DMSO and TMA*N*-O

Under conditions of reduced oxygen (sealed tubes) growth was stimulated (at least 10% greater optical density than the control) by addition of 3 g l^{-1} of KNO_3 in those strains able to reduce NO_3^- to NO_2^-. Only the growth of *D. aquamarina* and *D. venusta* was stimulated by the addition of DMSO or TMA*N*-O. Some strains (including ACAM 379) were able to reduce DMSO to DM_2S (dimethyl disulphide) and related compounds but produced less than 0.1 ppm DM_2S from 3 ml l^{-1} DMSO except for *H. halodurans* which produced large amounts of methanethiol in the headspace. The ability to reduce DMSO was not correlated with the ability to reduce NO_3^- to NO_2^-.

Under aerobic conditions ACAM 379 was able to grow using methionine as a sole carbon source, reducing this amino acid to DM_2S (identified, >1 ppm, in the headspace) (Figure 4.30).

4.7 Increase in tolerance to salt through uptake of proline

Proline was able to protect some colonies isolated from Ekho Lake against low water activity. After 1 week incubation, replicate plates of NA with 5% NaCl with or without 2% proline showed 20% more

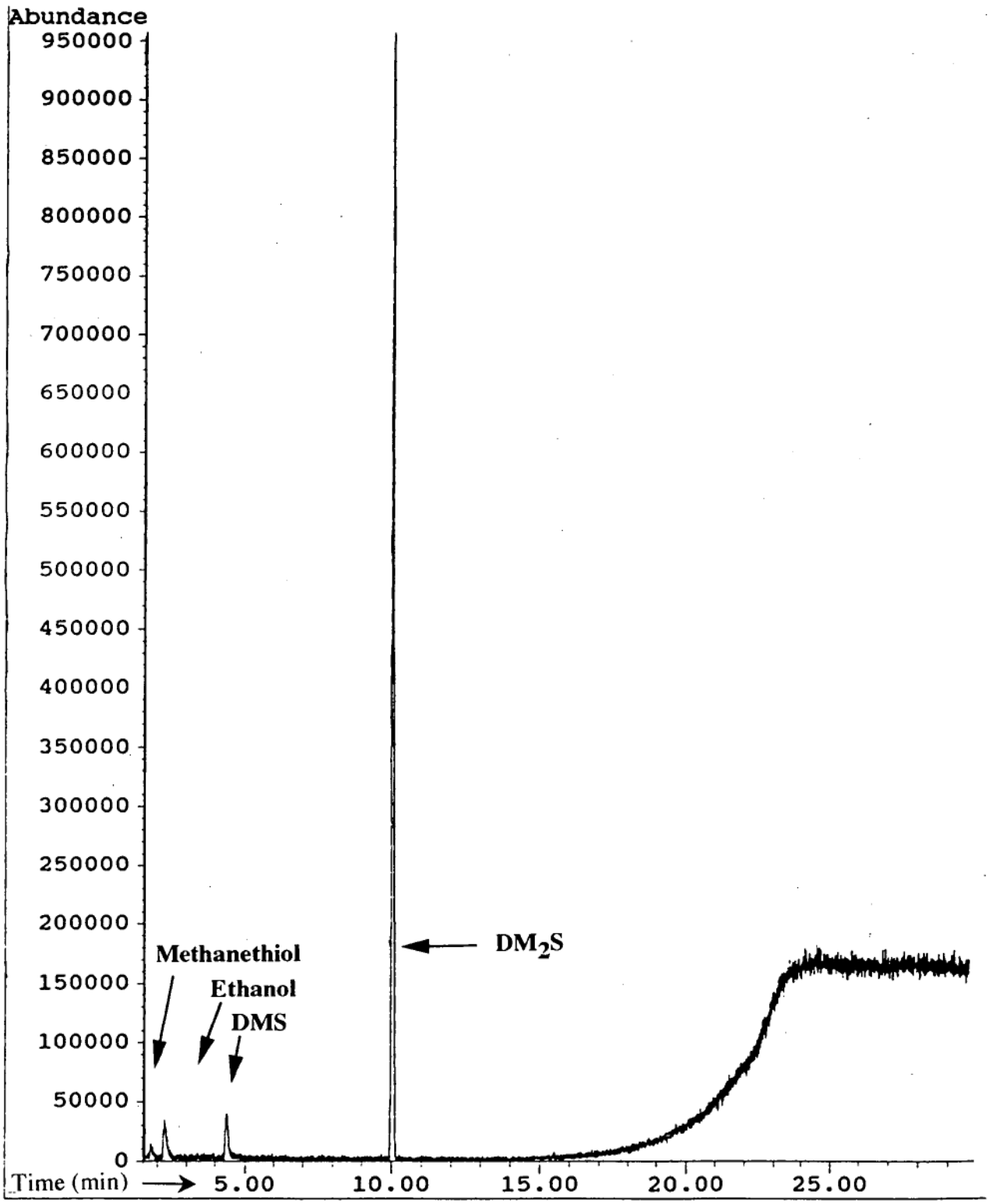


Figure 4.30: A GC-MS total ion chromatogram of head space of ACAM 379 grown using methionine as the sole carbon source. Peaks identified by mass spectrometry.

colonies on the plates supplemented with proline. NA with 8% NaCl supported growth of 8% more colonies if supplemented with 2% proline.

4.8 Taxonomy of novel species of *Brevibacterium.*

An extensive collection of bacteria from Ekho and Organic Lake isolated by P. Hirsch (University of Kiel) in 1992 was determined to consist mostly of members of the genera *Halomonas* and *Flavobacterium.* One isolate from the water column of Organic Lake was Gram-positive with a rod-coccus cycle (ACAM 480). From sediment collected from Organic Lake, E. Holdsworth (University of Tasmania, 1993) isolated another two similar strains: ACAM 379, 479.

As part of this study these three strains were characterised. Strain ACAM 379 had: distinctive rod-coccus cycle (Figure 4.31), yellow pigment, %mol G+C 62 (Figure 4.32), coryneform fatty acid profile (Table 4.3), MK 8(H2) major menaquinone and MK 7(H2) minor menaquinone, no significant ubiquinones (Figure 4.33) and a 16s rRNA sequence which grouped most closely with the *Brevibacterium* group (Figure 4.34, Table 4.4). Strain ACAM 480 (mol% G+C 58) was also shown to have MK 8(H2), MK 7(H2) menaquinone, yellow pigment and a rod-coccus cycle.

For ACAM 379 and ACAM 480 the notional minimum temperature for growth (T_{min}) was -2 °C and 0 °C respectively, the notional maximum temperature for growth (T_{max}) was 37 °C and the optimal temperature for growth (T_{opt}) 30 °C and 32 °C respectively (Figure 4.35.1). The nominal maximum %NaCl for growth ($Salts_{max}$) was 18% and the optimal %NaCl for growth ($Salts_{opt}$) was ≥0.1% (Figure 4.35.2). The minimum pH for growth (pH_{min}) was 4 and the optimal pH for growth (pH_{opt}) possibly between 5 and 8 (Figure 4.35.3). All strains had a yellow pigmentation which became pink on the addition of 1M NaOH (Jones & Keddie, 1986) indicating the strain may be related to the second homology group of *B. linens.*

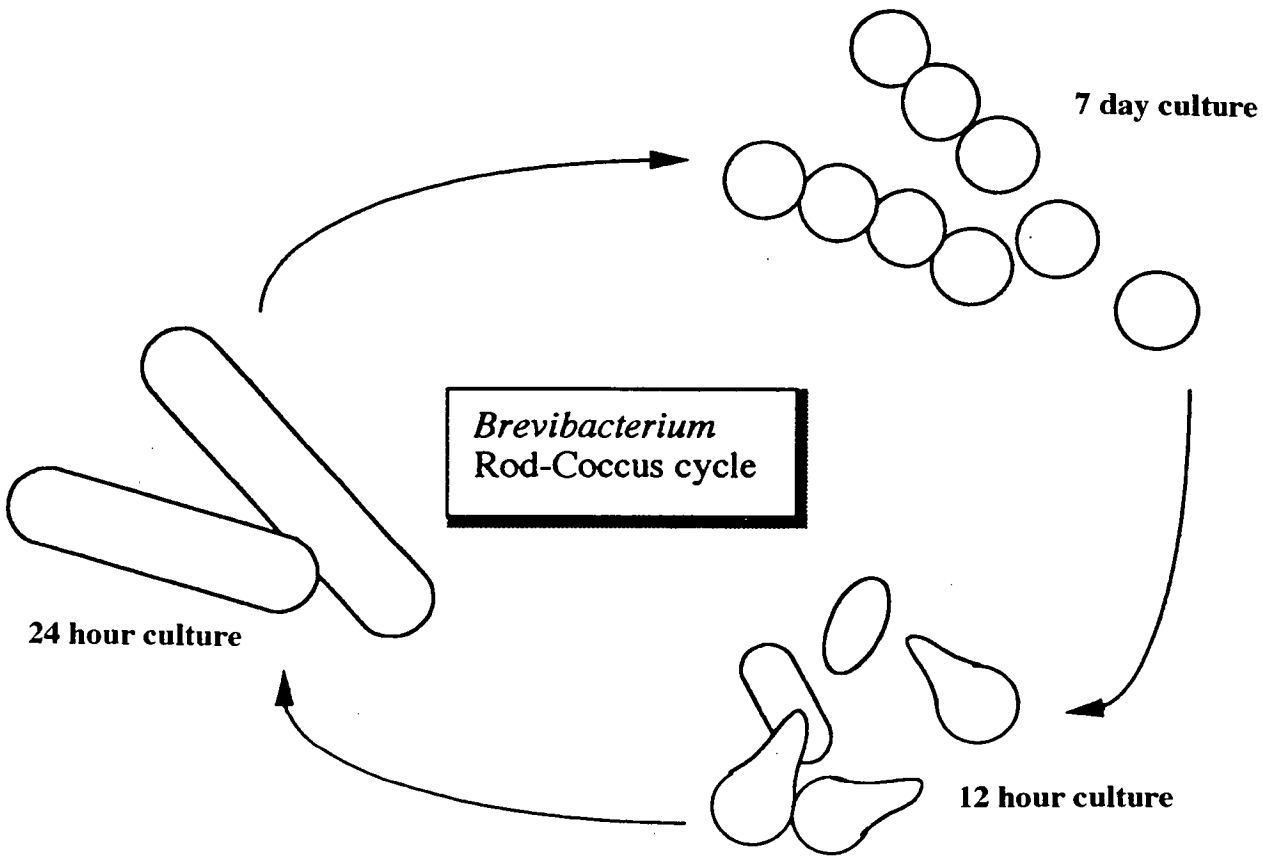


Figure 4.31: Illustration of the generalised *Brevibacterium* sp. rod/coccus cycle.

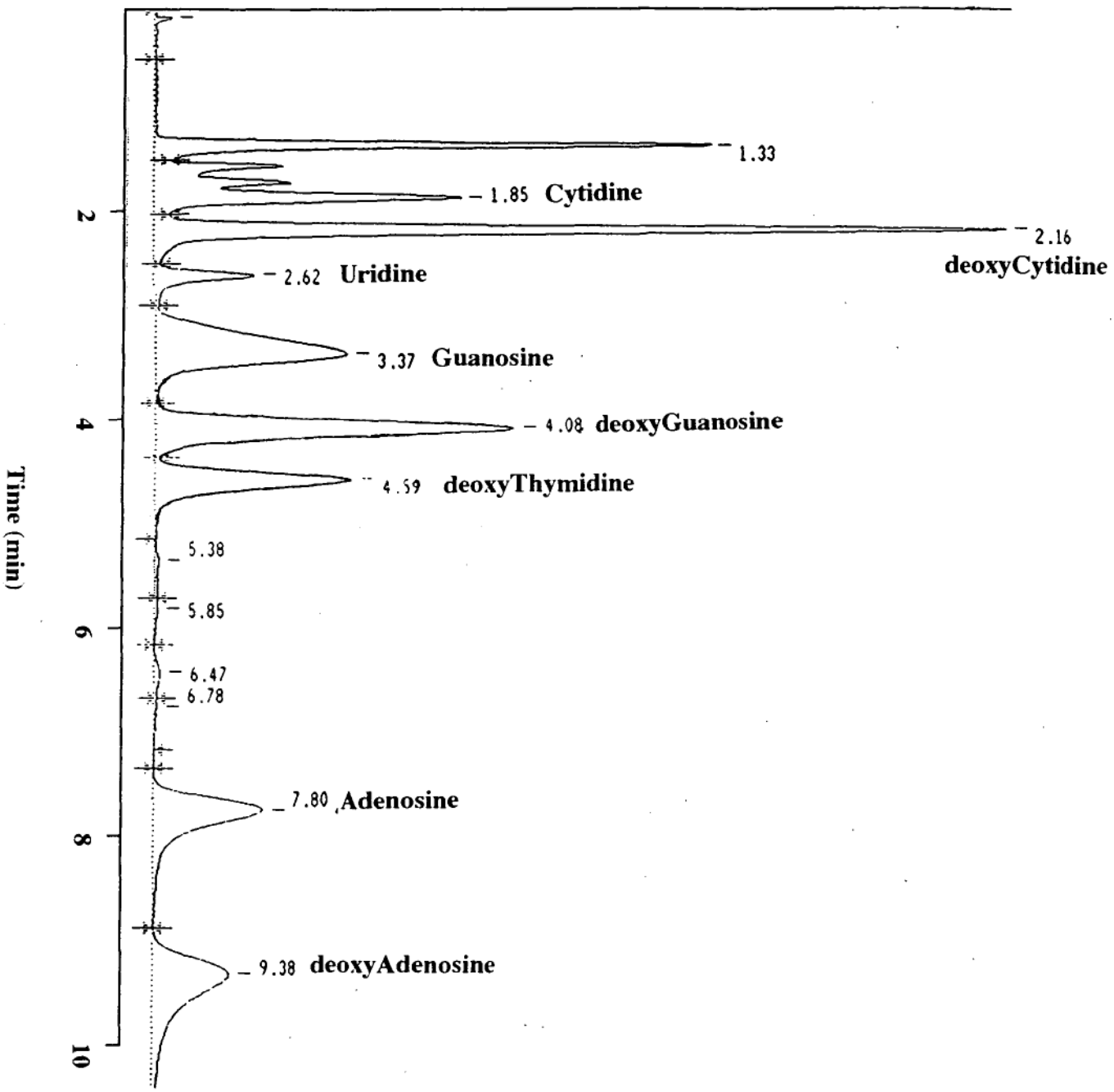


Figure 4.32: HPLC chromatogram of fragmented nucleosides of ACAM 379 DNA showing relative proportions of deoxycytidine, deoxyguanosine, deoxythymidine and deoxyadenosine also minor peaks of cytidine, uridine, guanosine and adenosine.

Table 4.3: Fatty acid profile for ACAM 379.

Sample Code: Fatty Acid	ACAM 379.
14:0	0.3
15:0	0.2
16:0	1.3
17:0	-
18:0	TR
Sum Saturates:	1.9
i14:0	1.1
i15:0	5.5
a15:0	57.5
i15:1	0.6
a15:1	1.0
i16:0	3.6
i16:1	6.1
i17:0	12.3
i17:1	10.5
Sum Branched:	98.1
18:1w9c	TR
Sum Monounsat.:	TR
Total:	100.0

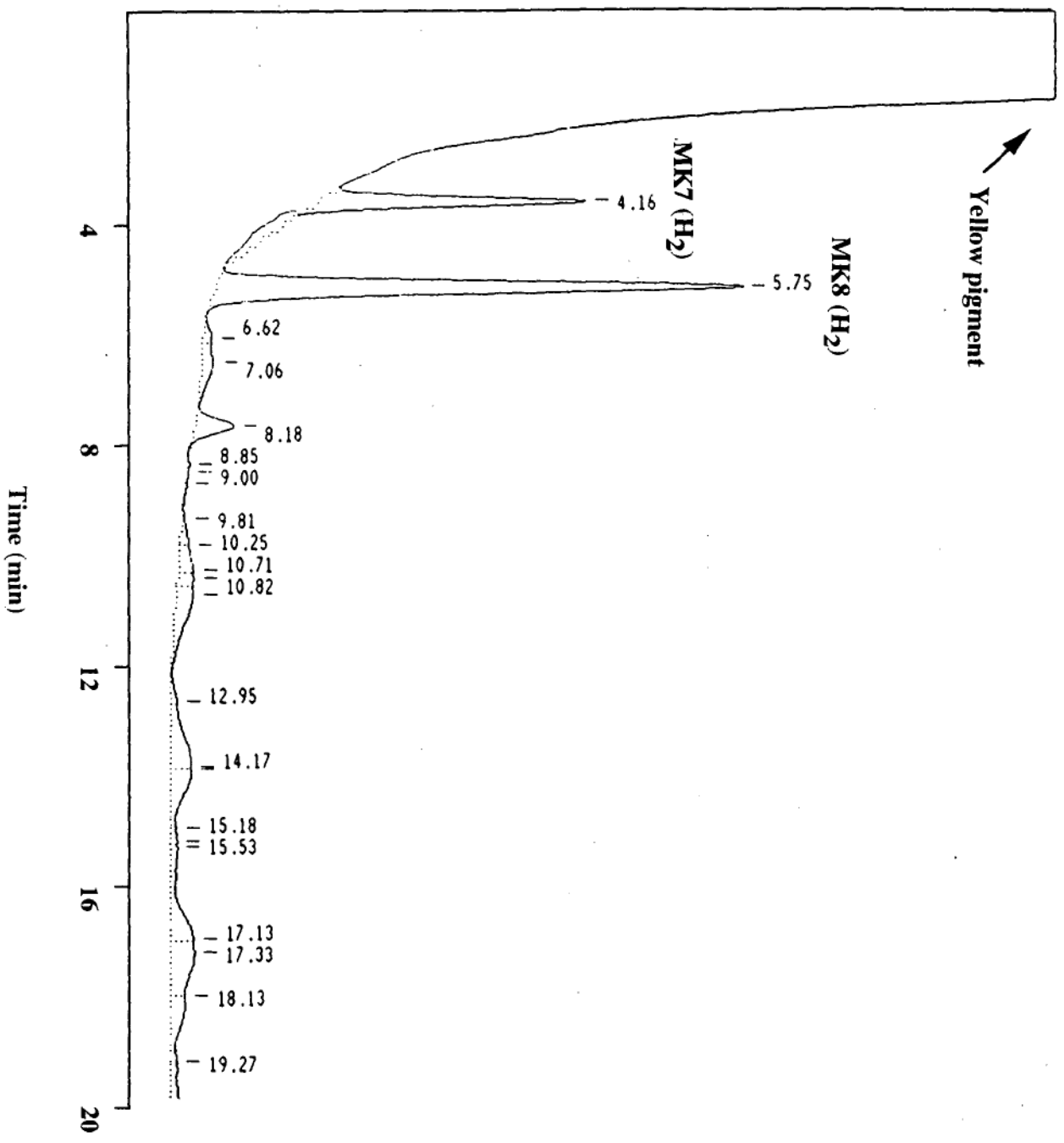


Figure 4.33: HPLC chromatogram of menaquinone extractions of ACAM 379 showing major menaquinones MK 7(H2) and MK 8(H2). The large initial peak was yellow pigment (not characterised).

0
UCCUGGCACAGAACGCUGGCUGCGUGCUUAACACAUGCAAGUCGAACGCU
GAAGCCCGCAGCUUGCUGUGGGUGGAUGAGUGGCGAACGGGUGAGUAACA
CGUGAGUAACCUGCCCCCGACUUCGGGAUAAGCCCGGGAAACUGGGUCUA
AUACCGGAUAUGACAACCGAAGGCAUCUUCGGUUGUGGAAAGUUUUUUCG
GUUGGGGAUGGGCUCGCGGCCUAUCAGUUUGUUGGUGAGGUAAUGGCUCA
CCAAGACGACGACGGGUAGCCGGCCUGAGAGGGCGACCGGCCACACUGGG
ACUGAGACACGGCCCAGACUCCUACGGGAGGCAGCAGUGGGGAAUAUUGC
ACAAUGGGGGAAACCCUGAUGCAGCGACGCAGCGUGCGGGAUGACGGCCU
UCGGGUUGUAAACCGCUUUCAGCAGGGAAGAAGCCCCUUGGGGUGACGGU
ACCUGCAGAAGAAGUACCGGCUAACUACGUGCCAGCAGCCGCGGUAAUAC
500
GUAGGGUACAAGCGUUGUCCGGAAUUAUUGGGCGUAAAGAGCUCGUAGGU
GGUUGGUCACGUCUGCUGUGGAAACGCAACGCUUAACGUUGCGCGUGCAG
UGGGUACGGGCUGACUAGAGUGCAGUAGGGGAGUCUGGAAUUCCUGGUGU
AGCGGUGAAAU**nnnn**AGAUAUCAGGAGGAACACCGGUGGCCAAGGCGGGA
CUCUGGGCUGUAACUGACACUGAGGAGCGAAAGCAUGGGGAGCGAACAGG
AUUAGAUACCCUGGUAGUCCAUGCCGUAAACGUUGGGCACUAGGUGUGGG
GGACAUUCCACGUUCUCCGCGCCGUAGCUAACGCAUUAAGUGCCCCGCCU
GGGGAGUACGGUCGCAAGGCUAAAACUCAAAGGAAUUGACGGGGGCCCGC
ACAAGCGGCGGAGCAUGCGGAUUAAUUCGAUGCAACGCGAAGAACCUUAC
CAAGGCUUGACAUACACCAGACCGGGCUGGAAACAGGUCCUCCUCUUUGA
1000
GGUUGGUGUACAGGUGGUGCAUGGUUGUCGUCAGCUCGUGUCGUGAGAUG
UUGGGUUAAGUCCCGCAACGAGCGCAACCCUCGUUCUAUGUUGCCAGCAC
GUGAUGGUGGGAACUCAUAGGAGACUGCCGGGGUCAACUCGGAGGAAGGU
GGGGAUGACGUCAAAUCAUCAUGCCCUUUAUGUCUUGGGCUUCACGCAUG
CUACAAUGGCUGGUACAGAGAGAUGCGAGACCGCGAGGUUUUAGCGAAUC
CCUUAAAGCCAGUCUCAGUUCGGAUCGUAGUCUGCAAUUCGACUACGUGA
AGUCGGAGUCGCUAGUAAUCGCAGAUCAGCAACGCUGCGGUGAAUACGUU
CCCGGGCCUUGUACACACCGCCCGUCAAGUCACGAAAGUCGGUAACACCC
GAAGCCGGUGGCCCAACCCUUGUGGAGGGGCCGUCUAAGGUGGACUGGCA
AUUGGGACUAAGUCGUAACAAGGUAGCCGUACCGGAAGGUGCGGCUGGAU
1500
CACCUCC

Figure 4.34: ACAM 379 16S rRNA sequence. The bold values **nnnn** are possibly GCGC (not determined due to compression). The sequence will be submitted to GenBank (Olsen *et al.*, 1990) on completion of the taxonomic paper (in preparation).

Table 4.4: A distance matrix formed from pairwise analysis of 16S rRNA similarity using the PAUP program (Phylogeny Analysis Using Parsimony) (Smithsonian Institute, 1993). The 15 closest relatives to ACAM 379 were selected from the Ribosomal Database (GenBank; Olsen *et al.*, 1991). *E. coli* was included as an outlier.

Below diagonal: Absolute distances
Above diagonal: Mean distances (adjusted for missing data)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1 *E. coli*	-	0.228	0.231	0.219	0.233	0.239	0.226	0.230	0.228	0.218	0.235	0.230	0.241	**0.250**	0.264	0.227	0.251
2 *Dermatophilus congolensis*	329	-	0.073	0.077	0.083	0.076	0.094	0.080	0.092	0.077	0.106	0.074	0.102	**0.102**	0.118	0.074	0.113
3 *Micrococcus luteus*	342	107	-	0.018	0.064	0.061	0.083	0.072	0.079	0.080	0.107	0.040	0.106	**0.099**	0.121	0.077	0.111
4 *Arthrobacter* sp. H.	196	69	16	-	0.068	0.071	0.083	0.079	0.080	0.078	0.112	0.047	0.098	**0.099**	0.097	0.084	0.100
5 *Renibacterium salmoninarum*	335	120	93	61	-	0.077	0.096	0.089	0.099	0.097	0.128	0.047	0.106	**0.109**	0.125	0.088	0.121
6 *Rothia dentocariosa*	345	111	89	64	112	-	0.091	0.086	0.094	0.085	0.118	0.061	0.097	**0.102**	0.111	0.087	0.110
7 *"Flavobacterium" marinotypicum*	326	137	121	75	140	133	-	0.024	0.058	0.085	0.120	0.082	0.113	**0.109**	0.134	0.065	0.124
8 *Aureobacter* sp.	332	117	106	71	129	126	35	-	0.053	0.080	0.112	0.079	0.111	**0.105**	0.127	0.061	0.119
9 *Clavibacter xyli*	341	134	118	72	143	137	85	77	-	0.081	0.106	0.080	0.105	**0.106**	0.132	0.062	0.118
10 *Terrebacter tumescens*	299	107	111	70	134	119	118	112	113	-	0.107	0.074	0.106	**0.094**	0.106	0.079	0.099
11 *Arthrobacter simplex*	342	155	159	101	185	173	175	164	157	149	-	0.107	0.136	**0.132**	0.147	0.120	0.140
12 *Arthrobacter globiformis*	345	108	61	42	68	90	121	116	121	104	159	-	0.100	**0.096**	0.117	0.082	0.106
13 *Brevibacterium linens*	348	146	154	88	150	140	162	160	154	148	196	147	-	**0.042**	0.047	0.118	0.054
14 ACAM 379	**368**	**146**	**145**	**89**	**155**	**147**	**156**	**150**	**157**	**131**	**191**	**143**	**62**	**-**	**0.064**	**0.106**	**0.045**
15 *Brevibacterium epidermidis*	384	169	176	87	176	159	191	181	194	147	212	174	69	**94**	-	0.133	0.022
16 *Brevibacterium halotolerans*	326	106	112	75	125	125	93	87	90	110	172	119	171	**154**	193	-	0.128
17 *Brevibacterium casei*	357	161	159	90	171	157	177	169	170	137	200	153	78	**65**	32	185	-

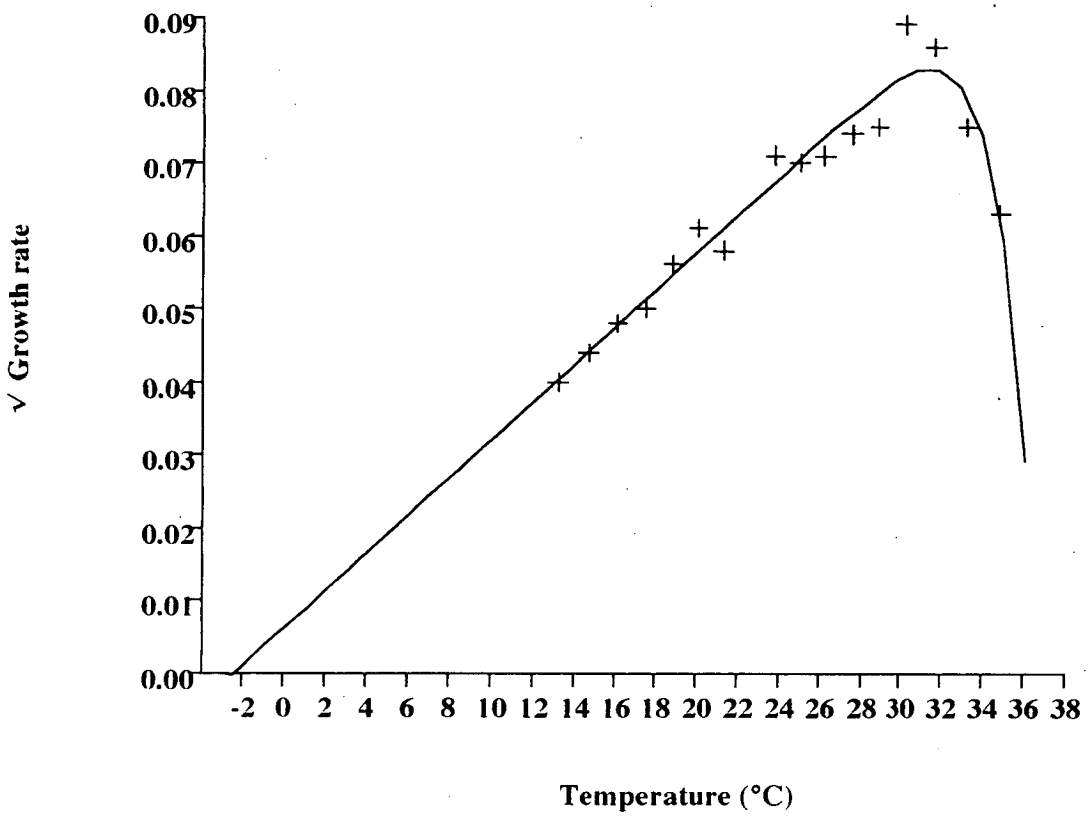


Figure 4.35.1: Square root of rate of growth versus temperature for ACAM 379. T_{min} and T_{opt} estimated by the four parameter square root model of Ratkowsky *et al.* (1983).

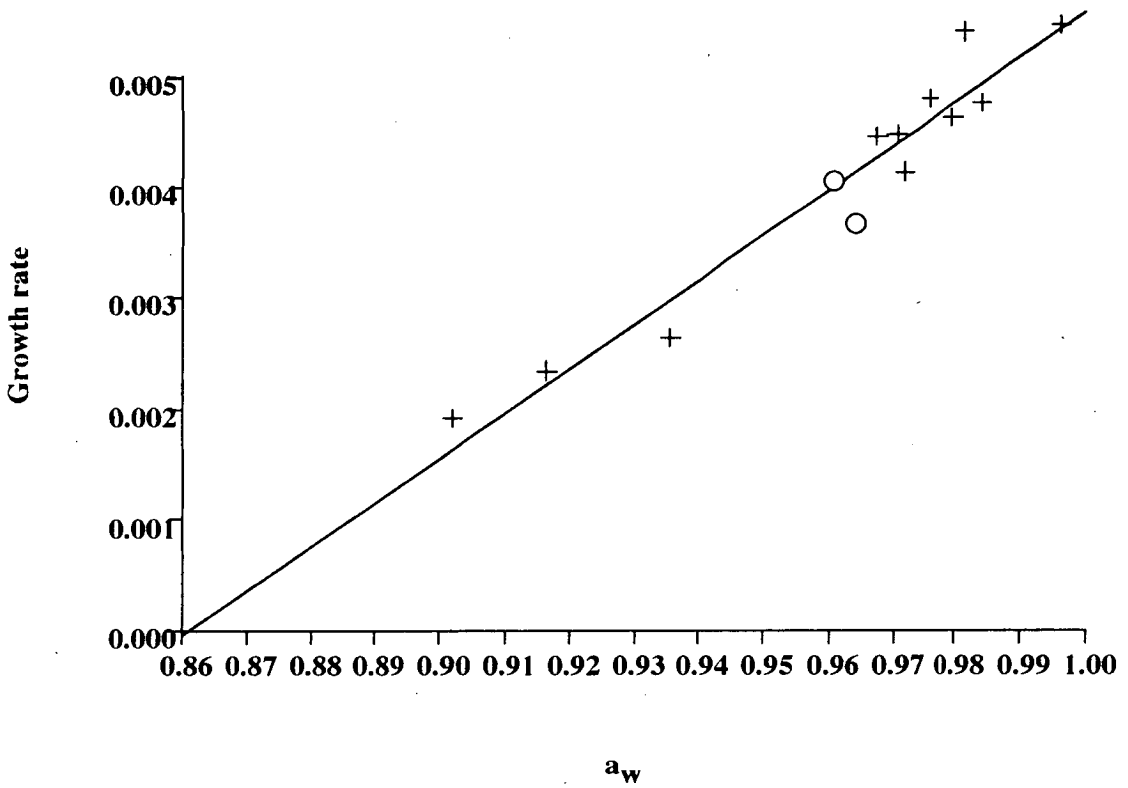


Figure 4.35.2: Rate of growth versus a_W for ACAM 379 extrapolated by a linear regression. Cross symbols indicate a_W was adjusted by NaCl addition. The open circles indicate a_W was adjusted by addition of AOLS (AOLPYA without the peptone, yeast or agar Appendix 1.2.1).

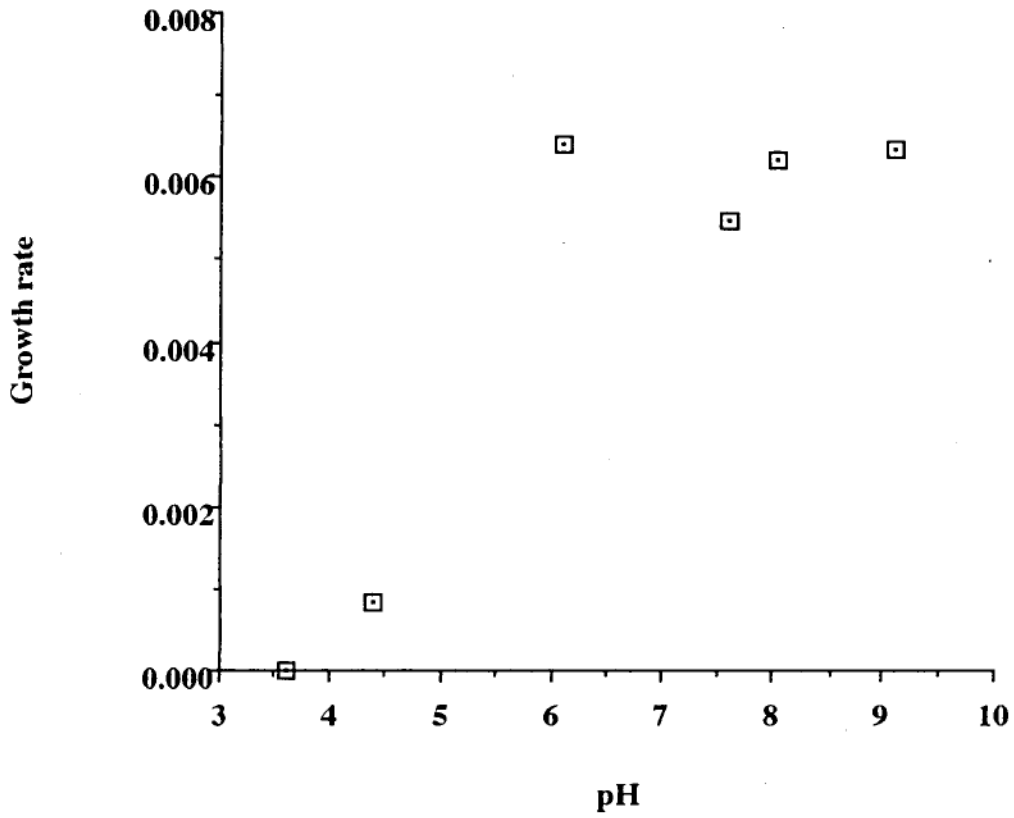


Figure 4.35.3: Rate of growth versus pH for ACAM 379.

5. DISCUSSION

5.1 Background to lake biota

Only *Halomonas* spp. and *Flavobacterium* spp. have been consistently isolated from the oxylimnion of Organic Lake and Ekho Lake on nutrient supplemented lake water media (Franzmann *et al.* 1987b; Garrick & Gibson 1988). Using a variety of media, Hirsch & Siebert (1991a) isolated other bacterial taxa from these lakes. A small number of strains probably belonging to the genera *Planctomyces* (6 isolates), *Pirellula* (4 isolates), *Gemmata* (2 isolates), *Hyphomicrobium* (2 isolates) and *Blastobacter* (2 isolates) were isolated. Some others were likely to be members of the genera *Bacillus*, *Vibrio* and *Spirillum* (Hirsch & Siebert 1991a). However most isolates belonged to the genera *Halomonas* and *Flavobacterium*. This reduced diversity is characteristic of harsh environments (Alexander, 1976).

All the lakes sampled supported microalgal populations, most commonly *Tetraselmis* spp. (Hirsch & Siebert, 1991b) and coloured flagellates and dinoflagellates (J. van den Hoff, Pers. Comm., 1994). Soil run-off and melt waters associated with the summer thaw provide additional nutrient sources to the lake (Burton, 1981b).

5.2 Determination of species composition by immunofluorescence microscopy

Only *H. meridiana, H. subglaciescola, F. gondwanense* and *F. salegens* have been consistently isolated from the oxylimnion of the meromictic hypersaline lakes of the Vestfold Hills. Antisera raised against the type strains of these four groups were successfully used to quantify changes in species composition within these lakes. The technique provided specific information on the relationship between culturable species and *in situ* populations. This study supported the findings of other workers who have observed excellent strain specificity of antibacterial antibodies (Dahle & Laake, 1982).

5.2.1 Specificity of the antisera

Antibodies raised against most bacteria were strain or species specific.

Only *F. salegens* cross-reacted outside the species. *D. venusta* (DSM 4743), a marine bacterium, showed strong agglutination reaction with antisera raised against the type strain of *F. salegens.* In a detailed chemotaxonomic study of the family *Halomonadaceae*, Franzmann & Tindall (1990) demonstrated that the fatty acid composition of *D. venusta*, "... did not conform well to the general pattern of fatty acids possessed by other members of the family *Halomonadaceae.*" Franzmann & Tindall (1990) went on to suggest that *D. venusta* is a peripheral member of the family. Though this does not account for the strong agglutination reaction to antibodies raised against bacteria of a different genus, it does offer some explanation why *D. venusta* alone demonstrated this enigmatic reaction. Despite the close chemotaxonomic relationship reported between *H. meridiana* and *D. aesta* (Franzmann & Tindall 1990) no cross-reactivity was observed between either of the *Halomonas spp.* antisera and any of the *Deleya spp.* tested, including *D. aesta* or *D. aquamarina* (now recognised as synonymous with *D. aesta*; Akagawa & Yamasato, 1989).

Bacteria from the more saline lakes, such as *H. subglaciescola*, swell and undergo other morphological changes when grown in conditions of lower salinity (James, 1990). This may affect the cell membrane and, in turn, the antibody binding sites. In this study bacterial antigens were grown in media closely duplicating the lake conditions. 1/2AOLPY broth (Appendix 1.2.1) (60 ‰ TDS) was considered at the start of this study to represent about the average salinity at which the isolates would be found. The four serogroups were found *in situ* between 61 ‰ and 165 ‰ TDS. *Halomonas* sp. strains grown between these salinities were tested in the laboratory and showed no decrease in antibody specificity.

5.2.2 Distribution of test species

All four bacterial species used in this study, originally isolated from the oxylimnion of Vestfold Hills meromictic lakes with ≥147 ‰ TDS (Dobson *et al.,* 1991; Garrick & Gibson, 1988), were shown by this study to occur in lakes where the TDS was between 61 ‰ (Fletcher Lake) and 165 ‰ (Organic Lake) and temperature between -10 °C (Organic Lake) and +18 °C (Ekho Lake).

This study has shown a changing population of bacterial species in the three most saline lakes under observation. No standard statistical

techniques may be applied to these data due to the inevitable interdependency of results. Observational interpretation has confirmed that bacterial populations do vary with season, depth and lake (Table 4.1; Figures 4.4 and 4.5). Both *H. subglaciescola* and *H. meridiana* serogroups have been shown to occur in Straight-In (Burch) Lake, Laternula Lake, Organic Lake, Ekho Lake and Fletcher Lake. Cemetery lake has an appropriate salinity for supporting *H. subglaciescola* and *H. meridiana*, but they were not observed in this site. Conditions within the lakes of the Vestfold Hills vary. DOC availability, temperature, haline composition and lake biota may all influence suitability of the lake for supporting the growth of *H. subglaciescola* and *H. meridiana* or any other bacteria.

F. gondwanense was most often found in the highest salinity lakes (Laternula Lake, Cemetery Lake and Organic Lake) though small populations were found in Ekho Lake and Fletcher Lake. The presence of *F. gondwanense* was significantly correlated with hours of sunlight (Figure 5.1). The lag in increase of *%F. gondwanense* of total bacterial count from August to November could be explained by the zone of thermal arrest where, because of the latent heat of ice, the heat from the sun goes to the melting of the ice with no rise in ice temperature. After November the ice cover thaws. This trend was borne out by colonies observed on lake water inoculated culture plates, where pigmented strains were more common during the summer. *F. salegens* was too poorly represented to determine any relationship with hours of sunlight. *F. salegens* was very poorly represented throughout the entire sampling period. *In vivo* it grew slower than *F. gondwanense*. Simidu *et al.* (1986) found that 88% of strains isolated from Antarctic surface seawater samples belonged to the *Flavobacterium-Cytophaga* complex. In deeper waters (>150 m) non-pigmented strains predominated. Simidu *et al.* (1986) support the proposition that the pigmented bacteria have a close association with marine algae which dominate the upper waters. However, in this study the presence of *Flavobacterium* spp. did not correlate with chlorophyll concentrations.

In this study it was found that up to 2% of the total bacterial population of the lakes were autofluorescent or stained non-specifically. This finding is similar to observations by Hicks *et al.* (1992) who found between 0.1 and 6% of the bacterioplankton in their samples were

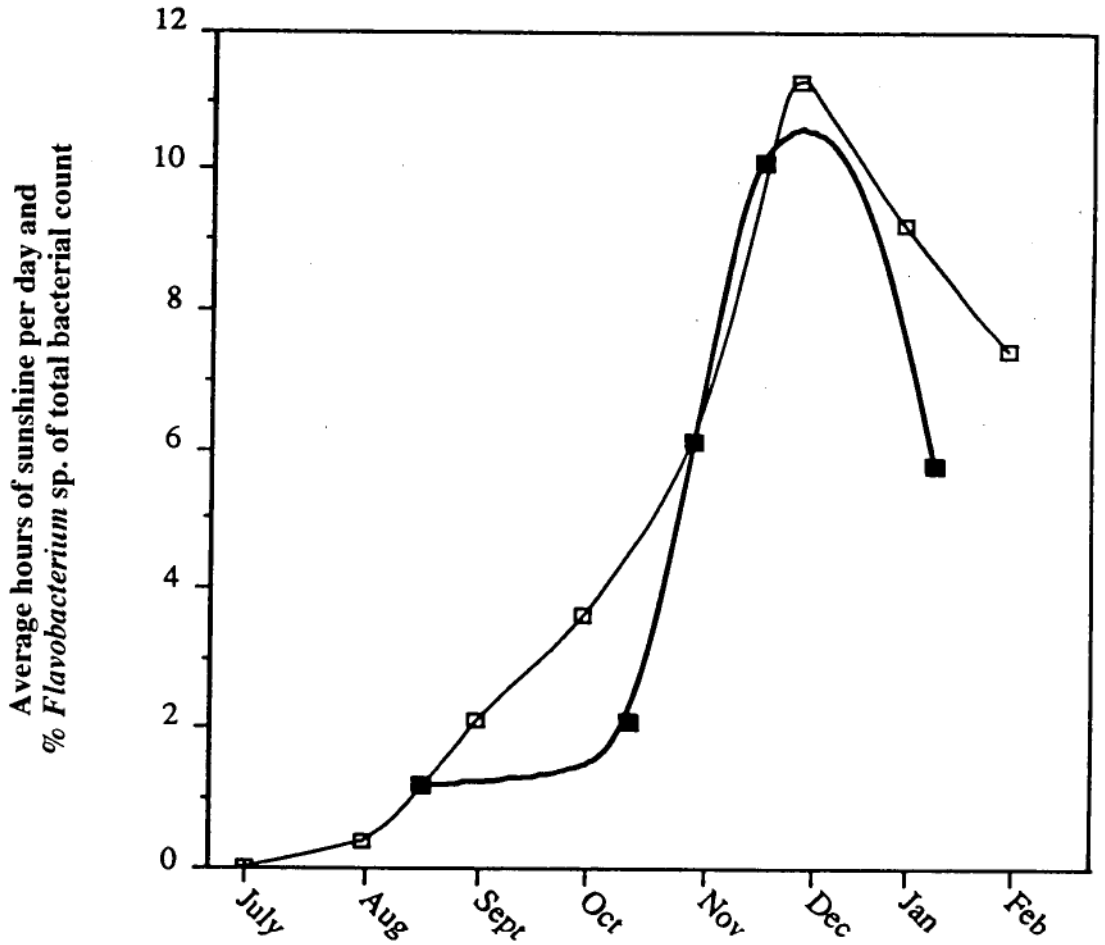


Figure 5.1: Average hours of sunshine per day (-□-) (from monthly meteorological reports) and % *F. gondwanense* of total bacterial count (-■-), from Organic Lake at 2 metres depth from July 1990 to February 1991.

autofluorescent and may have been mistaken for cells which had bound with fluorescent probes.

Dahle & Laake (1982) presented a substantial work on the abundance in seawater of five species isolated from seawater. They generated specific antibodies to an *Aeromonas* sp., *Chromobacterium lividum*, a *Vibrio* sp. and two *Pseudomonas* spp.. Growth of the five strains, isolated on high nutrient media, was stimulated by high values of primary production, chlorophyll and DOC. Dahle & Laake (1982) found a general increase in immunologically identified strains during a small initial increase in primary production and again during a second algal bloom where primary production reached its maximum. This result closely parallels trends seen in this study. Because of their ability to grow rapidly on high nutrient media it would be expected that the bacteria used in this study would be primarily associated with situations of high nutrient input into the lake ecosystem. The strains quantified in this study were isolated with media containing labile substrates in the order of g l^{-1}.

During the winter sampling period (from July 1990 until August 1990) none of the four serogroups were observed in numbers greater than 0.1% of the total bacterial count despite lake conditions often being within the experimentally determined temperature and salinity ranges for the four original strains (Table 5.1). Throughout the whole season all but *H. subglaciescola* were present only at salinities above their experimentally determined optimum (Table 4.1). Most isolates from continental Antarctic environments exhibit a higher optimum growth temperature than the *in situ* temperature (Franzmann & Dobson, 1993). This may indicate selective pressures in the other more optimal environments, although it is not necessary for bacterium to be functioning at maximal rates as long as it can compete effectively (Russell, 1990). In an environment where nutrient supply may be seasonal or limited, maximal metabolism would quickly exhaust the nutrient supply (Ellis-Evans, 1985).

5.2.2.1 Considerations for future species characterisation

Although species used to raise the four serogroups comprised the majority of isolates on nutrient supplemented lake water media, larger populations of unidentified, uncultured bacteria dominate the aerobic

Table 5.1: A list of *Halomonas* and *Flavobacterium* strains used to raise antibacterial antibodies, their temperature and total dissolved salts growth range and growth optimums as determined by growth in artificial broth.

Organism	Strain	Temperature (°C)		Salinity (‰)	
		range	optimum	range	optimum
H. meridiana	ACAM 246[a]	0 to 45	28 to 40	0 to 250	10 to 30
H. subglaciescola	ACAM 12[b]	-3 to 32	24	4 to 290	80 to 100
F. gondwanense	ACAM 44[c]	-6 to ≥25[d]	≈18	0 to ≥150[e]	50
F. salegens	ACAM 48[c]	-5 to ≥25[d]	≈18	0 to ≥200[e]	50

[a] Also - ATCC 49692, NCIMB 13119; Data from James *et al.* (1990).

[b] Also - ATCC 43668, DSM 4683; Data from McMeekin & Franzmann (1988) and Dobson (1988).

[c] Temperature data from closely related strains; Data from Dobson (1988).

[d] Cultures grew well at 25 °C but were not tested at higher temperatures.

[e] Cultures grew well at 150 and 200‰ respectively but were not tested at higher salinities.

waters of the lakes for most of the year. So the observation that only *H. meridiana, H. subglaciescola, F. gondwanense* and *F. salegens* have been consistently isolated from the oxylimnion of the meromictic hypersaline lakes of the Vestfold Hills reflects the limitations of cultural techniques, rather than these being the only bacteria present. Immunofluorescence techniques can only partly resolve the level of Antarctic microbial diversity. Recent molecular approaches may improve the situation dramatically. Moyer *et al.* (1994) have used restriction fragment length polymorphism (RFLP) to obtain data on the number of populations within a bacterial community and the genetic relatedness of those populations. Fani *et al.* (1993) used random amplified polymorphic DNA (RAPD) to obtain probes for bacterial identification which were specific to genus, species and strain. These were successfully applied to the *in situ* community. RAPD probes are presently being developed at the University of Tasmania to examine communities of the hypersaline lakes of the Vestfold Hills.

Careful consideration should be given to the species of bacteria chosen for future work. Bacterial strains isolated on low nutrient media or media which closely duplicates the lake conditions have been under represented. Enriched medium, salt reduced medium and incubation at abnormal temperature may all select for opportunistic strains which would normally only be present in the natural environment in very small numbers. On the other hand the levels of productivity were highest after the summer thaw and algal bloom, i.e. when the water column was nutrient enriched with reduced salt concentrations and higher temperature. These opportunistic strains, normally only present in very small numbers, may carry out most of the turnover in the lake environment (Section 5.3.2).

Immunofluorescence methods may also label dead or metabolically inactive cells. In the cold brines of hypersaline lakes it is not known how quickly dead cells are scavenged from the environment. Immunofluorescence combined with a bacteria specific indication of metabolic activity such as autoradiography would be an ideal way to overcome this problem (Fliermans & Schmidt, 1975).

5.3 Measurement of microbial activity by radioisotope incorporation

5.3.1 Determination of optimum assay conditions

5.3.1.1 Conversion factors

The validity of using a single conversion factor, theoretically or empirically derived, for the determination of bacterial growth rate from Tdr incorporation across systems is contested by Chrzanowski *et al.* (1993). They noted that only simultaneously determined empirical conversion factors gave growth estimates which were similar to those obtained from changes in cell abundance. They conclude that the use of any other conversion factor will give considerable variation in estimated growth rates. For example, calculation of N assumes that the mol% G+C of the heterotrophic population is 50. In Ekho Lake during January at 16 m depth at least 40% of the population was found to be from the genus *Halomonas* which has a mol% G+C of about 60. In this case N would be underestimated by about 8%. Values converted from incorporation data (N, g and C) are only estimations and should be interpreted with care.

On the other hand, Bell (1990) concluded that variation of conversion factors from the theoretically derived value of $1\text{-}3 \times 10^{18}$ cells mol^{-1} is simply the result of unaccounted dilution. In this study, a theoretically derived conversion factor of 5×10^{17} cells mol^{-1} (Moriarty, 1990) was used. As literature values vary between 0.2 and 68×10^{18} cells mol^{-1} (Mitchel & Bloem, 1993) care was taken comparing data from this study with literature generation times. Wherever possible rate of Tdr incorporation was quoted rather than converted data.

5.3.1.2 Incubation time

Incubation times of up to 36 hours have been used for Antarctic marine samples (Robarts & Zohar, 1993). Normally only 15 minutes is necessary for temperate marine samples, or up to 2 hours for samples from colder waters (Grossmann & Dieckmann, 1994; Moriarty, 1986). Two hours proved sufficient for all sites analysed in this study. Figure 4.10 shows a time course study over the range of depths in Ace Lake. In all cases the incorporation of Tdr was approximately linear at 2 hours incubation.

5.3.1.3 Saturation of radioisotope incorporation

In most studies of aquatic environments 5 to 10 nM of Tdr has proved sufficient to saturate the sample (Bell, 1986). In the analysis of soil samples, workers have used up to 200 nM of Tdr (Bååth, 1994). When analysing solar salterns, Oren (1990) saturated the system with 50 nM of Tdr. Moriarty (1990) suggested 20 nM as suitable for most water bodies and 50 nM if detritus was present. In this study, 48 nM of Tdr proved sufficient to saturate the system in all cases.

For Leu incorporation, maximal incorporation rate can be extrapolated by Michaelis-Menten kinetics from plots of Leu incorporation rate against concentration of added Leu (Kirchman *et al.*, 1986; Servais, 1995). Insufficient data points were collected in this study to apply this non-linear model with confidence. Figure 4.12 shows the data plotted with polynomial extrapolation. Conservative estimates would suggest that the Leu concentration used in this study underestimated V_{max} by about 2 to 3 times. van Looji & Riemann (1993) noted that published isotope dilution factors for Leu incorporation range from 1 to 11.8. This 'isotope dilution' should not be confused with the definitions of isotope dilution of Tdr (Moriarty, 1990). van Looji & Riemann (1993) plotted Leu incorporation rate against concentration of added Leu and defined dilution as the ratio between V_{max} and V for the Leu concentration used. In the case of the addition of 10 nM of Leu they list literature values between 1 and 5. These values bound the estimate used in this study.

5.3.1.4 Radiolabelled isotope dilution

Isotope dilution by extracellular and intracellular pools of thymidine may cause underestimation of DNA synthesis. Isotope dilution analysis gives radioisotope assay real relevance. It is a technique to measure the influence of diluting factors on DNA incorporation. Possible sources of isotope dilution are graphically illustrated in Figure 2.5. Following the techniques of Moriarty (1990), different amounts of unlabelled precursor (thymidine or leucine) were added with a constant amount of radiolabelled precursor. A plot of the reciprocal of isotope incorporation against the total amount of precursor (labelled and unlabelled) was extrapolated to give the amount of isotope dilution.

When the protocols used in this study were tested in temperate seawater, isotope dilution plots were consistently linear. Isotope dilution data collected in Antarctica were not analysed until return to Australia when further experimentation was not possible. These plots were found to be non-linear. Insufficient data were collected to truly determine the multiphasic or non-linear nature of isotope dilution plots derived in this study, the irregularities were such that these correction factors were not used in calculations (Figure 4.13). Other authors have found that the assumption of linearity in isotope dilution plots may be false (Pollard & Moriarty, 1984; Riemann *et al.*, 1982). Possible reasons may include multiphasic uptake kinetics or multiple intra- or extra-cellular pools. High levels of variation were found in all of the isotope dilution experiments in this study. This finding is supported by García-Cantizano *et al.* (1994) and Bell (1986) who have also published isotope dilution plots with high levels of variation, plots which did not lend themselves to linear interpretation.

Exogenous thymidine pools in aquatic systems are generally below 2 nM (Jefferey & Paul, 1988) though higher values have been reported for hypersaline waters. In solar salterns, Oren (1990) suggested that external thymidine pools ranged between 10 and 85 nM but did not use these correction factors in his analyses. Tibbles *et al.* (1992) found these pools to range from 0.3 to 9.3 nM in salt marsh lagoons. Evident in the literature and implicit in these discussions is the caveat that some of the conversion factors used in calculations have not been empirically tested in the cold hypersaline Antarctic environments. As more is understood about the Antarctic hypersaline lakes, more accurate productivity estimates can be calculated from existing incorporation data. If exogenous thymidine pools were of the order of 2 nM then reported productivity figures in this study would have been underestimated by 4%.

Simon & Azam (1989) found that in seawater ambient leucine concentration was generally less than 1 nM. A seasonal study by Gibson *et al.* (1994) reported that the concentration of leucine in Organic Lake oxic waters was below the limits of detection (0.01 μM) during spring and <1 μM during winter. Such exogenous pools of labile leucine would cause underestimation of Leu incorporation by <10%.

5.3.1.5 Temperature optima

Most bacteria isolated from the lakes of the Vestfold Hills grow optimally at temperatures above the temperature at which they were isolated (Franzmann & Dobson, 1993). *In situ* temperature in the natural environment is normally well below the optimal temperature for growth (Díaz-Ravina *et al.*, 1994).

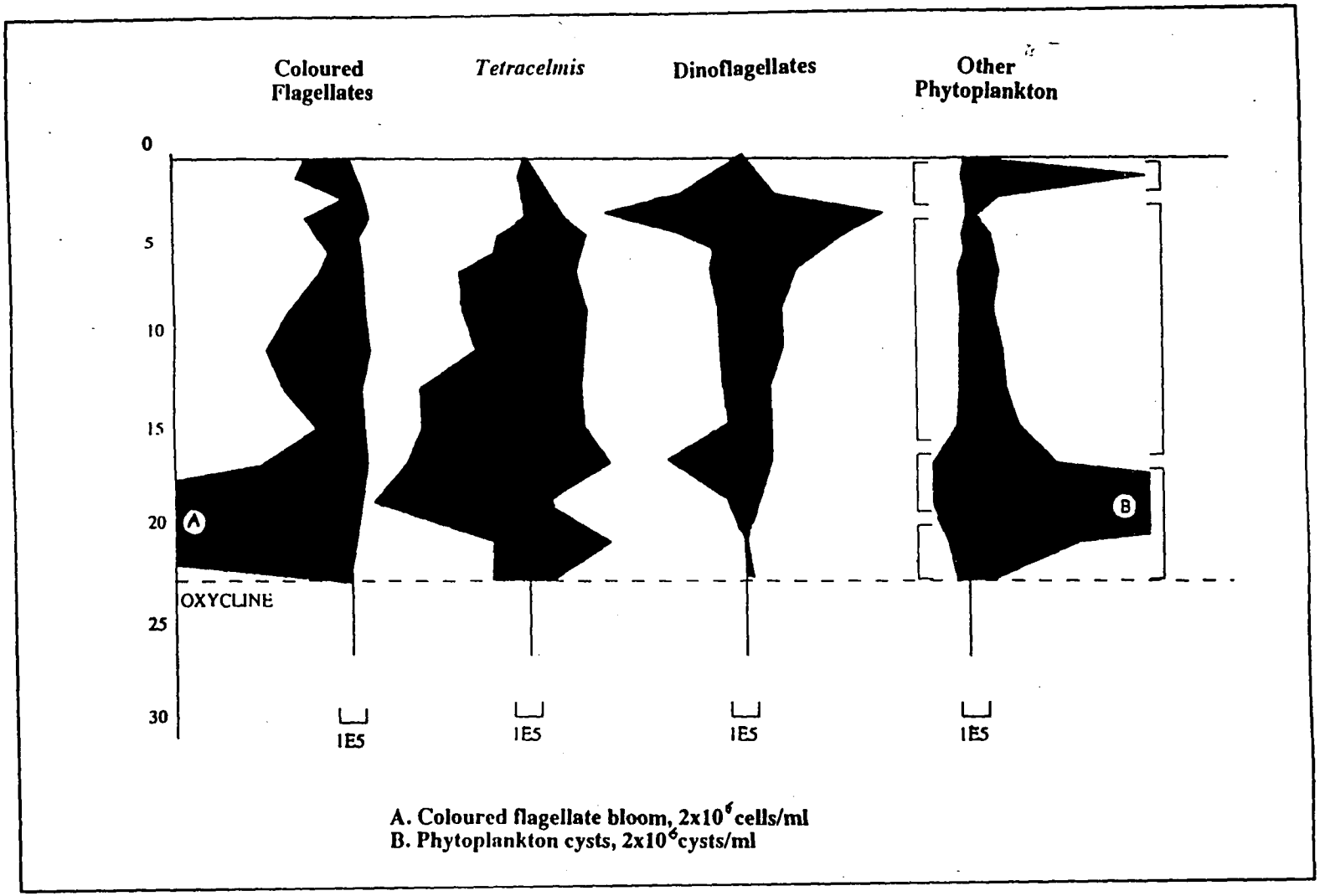
Herbert & Bell (1977) showed that maximal uptake of some substrates can be at *in situ* rather than optimal temperature. This is an important point, though bacteria show maximal productivity at 'optimal' temperature some physiological mechanisms operate optimally at different temperatures. Selective pressure would favour those bacteria which, at *in situ* temperature, had the highest affinity to and growth efficiency on the substrates which were available. Münster (1993) emphasised that the kinetic properties of enzyme systems associated with the capture and uptake of substrate must be adapted to suit *in situ* conditions. "To sustain growth rates the bacterial assemblage must continually alter its efficiency in utilising various types of substrate." (Sundh, 1992). The regulation of these uptake mechanisms is poorly understood (Münster, 1993).

The Tdr incorporation data from this study indicated bacterial productivity was maximal at about *in situ* temperature (Figure 4.14). Key to the interpretation of data from Tdr incorporation is the assumption that the incubation period is insufficient for the bacteria to adjust their metabolism to any change in environmental conditions. Though cells grow optimally (most rapidly) at higher temperature they are able to habituate to the low *in situ* temperature via physiological mechanisms which inhibit growth at higher 'optimal' temperature in the short term.

5.3.2 Seasonal variations in productivity

Though overall Tdr incorporation was low, a prominent summer and less obvious winter, peak was evident (Figure 4.16; Table 4.3) and were readily associated with environmental conditions. The dilution of surface waters during summer thaw, increase in water temperature, nutrient and free amino acid input from proliferation of algae in the upper waters (Figure 5.2) and increase in average hours of sunshine per day were

Figure 5.2: Total number of phytoplankton in the oxylimnion of Ekho Lake in December 1989 (left of median line) and January 1990 (right of median line). Determined by John van den Hoff (Australian Antarctic Division, Kingston, Tasmania).



A. Coloured flagellate bloom, $2x10^6$ cells/ml
B. Phytoplankton cysts, $2x10^6$ cysts/ml

coincident with fastest generation times. The less obvious winter peak was associated with a bacterial bloom in the anoxylimnion of all lakes in late winter. Burke and Burton (1988) noted a similar effect in Burton Lake (Salinity 44‰ salts) where they identified a bloom of bacteria in the anoxylimnion as photosynthetic *Chlorobium* spp., which grew in response to the return of light to the lakes. Recent work combining flow cytometry and microscopy has identified *Chlorobium* spp. in Ace Lake and Fletcher Lake and naturally fluorescing populations of unidentified bacteria in Ace Lake, Fletcher Lake, Ekho Lake and Organic Lake (Rankin & Pittman, 1992).

These seasonal variations in productivity in continental Antarctic lakes, with activity peaks in early summer, increased phytoplankton and bacterial activity observed during the height of summer and a general increase between these times, are typical of other Antarctic aquatic environments (Ellis-Evans, 1985; Knox, 1994; Nedwell *et al.*, 1993) and cold marine environments (Wikner & Hagström, 1991) and general marine systems (Albright & McCrae, 1987).

5.3.2.1 Comparisons with other environments

This section expands on data given in Table 5.2 which shows previously reported data for Arctic, Antarctic and hypersaline environments with equivalent data from this study. Lake Vanda is a meromictic hypersaline lake with virtually the same salinity profile as Fletcher Lake, 55‰ salts just above the oxycline rising to 121‰ salts at the sediment-water interface. In Lake Vanda the oxycline is at a depth of 59 m where the temperature was 20 °C (Wynn-Williams, 1990). Both lakes had maximum Tdr incorporation rates just above the oxycline in late summer. In Ekho Lake the temperature just above the oxycline in late summer was closer to the temperature in Lake Vanda but the salinity was 130‰ salts and the Tdr incorporation rate was only 0.4 pM h^{-1}. This suggests that increased salinity may limit incorporation rate. The incorporation results from Organic Lake and Ekho Lake were difficult to put into context as Tdr incorporation has not been measured in any similar hypersaline cold environment. Bloem *et al.* (1989) question whether extremely low generation times (in their study, 3 to 131 days) do represent growth or just biochemical turnover of nucleic acids. The Tdr incorporation rates from Organic Lake and Ekho Lake were within the general range indicated by temperature-dependency curves from

Table 5.2: Previously reported Tdr incorporation values and closest equivalent data from this study.

Study	Findings	Equivalent site	Data
Arctic seawater, March 1985 to May 1986 (Bunch & Harland, 1990)	0.01 mg C m^{-3} h^{-1}	Coastal marine site, June 1990, depth of 2 m.	0.03 mg C m^{-3} h^{-1}
Antarctic coastal seawater, January 1987, surface (Bailiff *et al.*, 1987).	0.17[a] mg C m^{-3} h^{-1}	Coastal marine site, January 1991, depth of 2 m.	0.13 mg C m^{-3} h^{-1}
Meromictic Lake Vanda, Wright Valley, Antarctica December 1980, just above oxycline (Vincent *et al.*, 1981).	2.5 pM h^{-1}	Fletcher Lake, January 1990, just above the oxycline.	3.6 pM h^{-1}
Solar saltern, Israel, July 1989, salinity 97‰, incubation at 18 °C[c] (Oren, 1990).	4 pM h^{-1}	Ekho Lake, January 1990[d], depth of 6 - 16 m, salinity 76‰; 18 °C.	0.3 - 1.1 pM h^{-1}
Solar saltern, Israel, July 1989, salinity 97‰, incubation at 8 °C[c] (Oren, 1990).	0 pM h^{-1}	Organic Lake, January 1990[d], depth of 2 m, salinity 114‰; 8 °C.	1.2 pM h^{-1}

[a] Recalculated using extrapolation factor of 5.1×10^{17}, as used in this study.

[b] Salinity 10‰ in post-thaw (January) surface waters, this lake is only hypersaline in the anoxylimnion.

[c] From temperature-dependency plots.

[d] Closest equivalent due to maximal sunlight in the water column (post thaw).

solar salterns of similar salinity (Oren, 1990). Generation times in Israeli solar salterns (90 to 400 ‰ salinity) were in the range of 1.1 to 22.6 days at *in situ* temperature (Oren, 1990). These times were much faster than Antarctic lake environments of similar salinity but lower temperature (Table 5.2; Figure 5.3). Oren (1990) refutes the statement by Rodriguez-Valera (1988) that "...growth rates of halobacteria are probably extremely low under the conditions of the most concentrated brines", however this observation seems to hold for the cold concentrated brines of Antarctica. Most Antarctic lake heterotrophic bacteria cultured so far have optimum growth temperatures in the range 22-34 °C and grow slowly at the *in situ* temperatures found in Antarctic waters (Franzmann & Dobson, 1993). Generation times in Ace Lake corresponded to rates from the coastal marine site and previously reported rates for Arctic and Antarctic marine ecosystems (Bailiff *et al.*, 1987; Bunch & Harland, 1990).

In Figure 5.3 the incorporation rates of adapted consortia from different environments of the same salinity (solar salterns and Organic Lake) fall on the same sigmoidal curve. The fastest growing organism under a particular set of conditions would be expected to come to dominate the microbiota in that environment so the observed growth rate for a mixed population will be that of the fastest growing strain or organism in the population (McMeekin & Ross, 1996). The incorporation rates for the solar salterns and Organic Lake may represent an upper limit to community growth rate at 23% salts. Díaz-Ravina *et al.* (1994) have found that the relationship between Tdr incorporation and Leu incorporation and temperature in soil communities conformed to the square root model of Ratkowsky *et al.* (1982) ($r^2 \geq 0.98$):

$$\sqrt{\text{Tdr incorporation}} = b(T - T_{min})$$

where **b = slope**
T = temperature

The data of Oren (1990) and this study was fitted to the square root model and the nominal T_{min} for incorporation at 23% salts was 3.7 °C (r^2=0.72). Tdr incorporation at ≥23% salts was observed below 3.7 °C but in all cases this incorporation was less than 0.2 pM h^{-1}.

The generation time in the Baltic sea where temperatures can be as low as 1 °C can be 1 or 2 days. Heinänen & Kuparinen (1991) concluded

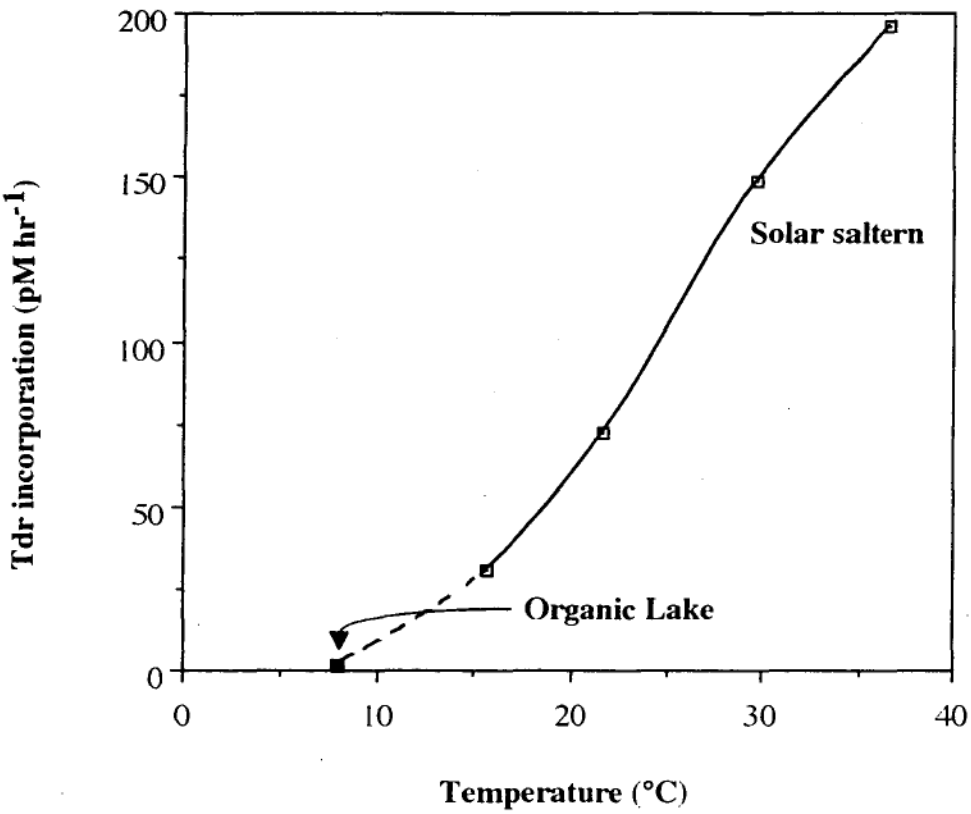


Figure 5.3: Tdr incorporation rate versus temperature for solar salterns (adapted from Oren 1990). Maximum Tdr incorporation rate in Organic Lake (January, 2 m depth) is included for comparison.

from this that high frequency sampling is needed once active growth periods are identified. They noted average Tdr incorporation in the area of the Baltic sea of 3 to 32 pM h^{-1}. During peak bacterial activity in this study (2 m depth, January) Tdr incorporation was up to 5 pM h^{-1} and generation times as low as 9 days. Higher frequency sampling (greater than bimonthly) is strongly indicated in this system as well.

Jonas *et al.* (1988) listed Tdr incorporation in various aquatic systems as varying from 0.02 to 635 pM h^{-1}. Tdr incorporation was of the order of 40 to 85 pM h^{-1} in a number of Swedish lakes (Bell, 1986). In this study Tdr incorporation was between 0.01-5 pM h^{-1}.

Tdr incorporation and Leu incorporation data will give an indication of increase in biomass, but not the rate of loss of biomass. A starving or surviving population, for instance, may consist of those bacteria which are dying and those which are growing on the nutrients released by those which are dying (cryptic growth). In which case radioisotope incorporation would give a misleading indication of the status of the community. Microautoradiography combined with vital staining would elucidate this situation (Tabor & Neihof, 1982).

5.3.2.2 Radioisotope incorporation by anaerobic bacteria and algae

The Tdr incorporation method may underestimate anaerobic bacterial growth. Tdr is not generally incorporated by photosynthetic bacteria (Pollard & Moriarty, 1984) or sulphate-reducing bacteria (Winding, 1992) which have been found in the anoxylimnion of meromictic lakes throughout the Vestfold Hills (Burke & Burton, 1988; Gibson *et al.*, 1991). Alternative measurements of bacterial activity show extremely slow rates of sulphate reduction (Franzmann *et al.*, 1988a; Gibson *et al.*, 1991) in continental Antarctic lakes. However, from the changes in total bacterial numbers observed in this study, parts of the bacterial assemblage in the anoxylimnion are capable of generation times of at least 69 days (production rates of at least 2.5×10^3 bacteria ml^{-1} h^{-1}) in Ekho Lake (July to September 1990, 22 m depth) and similar rates were implied in other lakes (Appendix 7). Investigations into the seasonality of bacteriochlorophyll concentration in the anoxylimnion of these lakes are currently being carried out on the assumption that the blooms are of photosynthetic bacteria. Work from the summer season 1991 (Rankin &

Pittman, 1992) has found that numbers of *Cyanobacteria* spp. peaked at 11 m in Ace Lake in December at $9x10^6$ cells ml^{-1}. *Chlorobium* spp. peaked at 12 m in Ace Lake in January at $8x10^7$ cells ml^{-1}. Similar results were found in this study. *Tetraselmis* sp., common to the oxylimnion of the hypersaline lakes (Hirsch & Siebert, 1991b), does not incorporate Tdr into DNA (Martinez *et al.*, 1989). The Tdr incorporation method may underestimate Tdr incorporation in the anoxylimnion of Antarctic hypersaline lakes. However, extrapolation below the oxycline (Figures 4.10, 4.14, 4.16, 4.17) was consistent with lower rates of bacterial activity observed by alternative measurements.

5.3.3 Correlations with other biological and physio-chemical factors

Abundances of the three species which demonstrated distinct seasonality peaked at various depths in December 1990. These maxima were coincident with the general summer increase in lake productivity (Figures 4.4, 4.5, 4.16.1 peak C).

The lack of direct correlation between total bacterial numbers and production or metabolic activity suggests that either there are a proportion of inactive (dead) cells, or there are large changes in the metabolic activity per cell which may be associated with numerous environmental conditions or with taxonomic succession (Delille, 1993).

Slezak *et al.* (1994) showed that incorporation of Tdr and Leu decreased with decreasing pH (acrylic acid) in marine samples. They found variation of 10% in the growth range between pH 8 and 7. Between pH 8 and 5, however, production and protein synthesis dropped by 60%. The growth rate of Antarctic isolates such as *Brevibacterium* spp. (Figure 4.35.3) and *Halomonas* spp. (James, 1989) also decreased below pH 7. As study sites had pH between 7.0 and 8.6; the general effect of pH was not considered as an influential factor in the analysis of variables. Weak (organic) acids may only be partly dissociated in the aquatic system. These acids can pass across the cell membrane and dissociate inside the cell. This action may inhibit the bacterial cell in addition to the general pH effect.

5.3.4 [^{3}H]leucine incorporation

Dry bacterial cell weight contains more than 50% protein so the rate of Leu incorporation into protein is a good estimate of growth rate. Various authors have found that Leu incorporation is: (i) strongly correlated to Tdr incorporation (van Looji & Riemann, 1993); and (ii) correlated with temperature i.e. that Leu incorporation decreased with temperature below 5 °C (Tulonen, 1993), or that there was a general decrease below 15 °C (Díaz-Ravina *et al.*, 1994). The Leu incorporation data from this study show no discernible trend (Figures 4.17, 4.18).

Figure 4.20.2 presents Leu incorporation plotted against salinity and temperature. Below 4% salts (1.03 g ml^{-1} salts) Leu incorporation was uniformly low. Leu incorporation increased above 4% salts while Tdr incorporation decreased markedly above 4% salts. Riemann & Bell (1990) state that, "Leu incorporation into protein may proceed even if net protein synthesis and cell production is zero." An increase in Leu incorporation may indicate that a larger portion of the population can take up Leu. Alternatively, under conditions of reduced labile DOC availability, cells may have a higher affinity for Leu, or an increased protein turnover.

Under optimal conditions, a rise in Tdr incorporation will be associated with a rise in Leu incorporation. As salinity increases and/or temperature decreases, a rise in Tdr incorporation is associated with a much greater rise in Leu incorporation (Synder *et al.*, 1994). As salinity and temperature change so dramatically over site, depth and time, the Leu incorporation data alone is difficult to interpret. However the systematic consideration of the ratio of Leu:Tdr makes this data more intelligible.

5.3.5 The ratio of [^{3}H]leucine incorporation to [methyl-^{3}H]thymidine incorporation

"Under conditions of balanced growth, the rates of macromolecular synthesis are coupled and the ratio of Leu:Tdr incorporation should remain relatively constant" (Tibbles *et al.*, 1992). van Looji & Riemann (1993) found a significant correlation between bacterial carbon production measured by Tdr and Leu incorporation (r^2=0.88, n=77). Analysis of pooled data from this study indicate that this is not always the case (r^2=0.01, n=60). Data showed variation in the ratio of Leu:Tdr

over site, season and depth. This would indicate changes in physiological state or perhaps in species composition, both of which are likely. This finding has since been supported by other workers in the Vestfold Hills lakes (J. Laybourn-Parry, Pers. Comm., 1995). In fact Hirsch and Siebert (1991a) have shown distinct variation of bacterial morphology with depth. Species abundance as indicated by immunofluorescence changes abruptly with depth and time in Organic Lake and Ekho Lake. Substantial decrease in the ratio of Leu:Tdr may also indicate cell division by non-growing cells. In this study the ratio of Leu:Tdr was correlated with DOC concentration (r^2=0.4) and the log of the generation time (r^2=0.4) but with no other biological, physical or chemical factor in the water column. Note that the DOC concentration may not indicate nutrient status as the percentage of labile or metabolisable carbon may differ from sample to sample (Section 5.5).

Literature values of the ratio of Leu:Tdr for aquatic systems vary from 1 to 39 (Section 2.4.3) but averaged around 10. In this study the average of data from samples which were >5 °C and <5% salts was 12±7, consistent with available literature and theoretical optimum values (see Section 2.4.3). The ratio of Leu:Tdr increased with increasing salinity and decreasing temperature to average maximum values of about 400. No harsher environment than these Antarctic sites have been investigated with this combination of techniques.

The maximum ratio of Leu:Tdr found in the literature was for studies of polar ocean populations. In cultures incubated at 1 °C in the dark, inoculated from the Weddell and Scotia seas, Bjørnsen & Kuparinen (1991) found ratios of Leu:Tdr between 10 and 180 in actively growing populations. As bacteria in the Weddell Sea sample stopped growing (after approximately 9 days), Leu incorporation remained the same for another day, increasing the ratio of Leu:Tdr to about 510. Growth appeared to peak, fall off, then peak again, indicating some unknown population interplay. Some of the change in incorporation characteristics were through species replacement, "...some selectivity and/or enrichment of the batches." (Bjørnsen & Kuparinen, 1991). A complicating factor was the unstable nature of the environment, introduced labile DOC, removal of predators and permanent darkness. Under the (relatively) stable conditions in the Antarctic sites, ratios were more likely to reflect the balanced 'optimum' for that environment.

The grouped ratio data for all sites plotted against salinity and temperature (Figure 4.21) conformed in a general way to the hypothetical model (Figure 2.2) introduced in the literature review (Section 2.2.2). A linear regression was plotted through data from sites with salinity more than 4% and temperature more than 5 °C to describe the increase in ratio of Leu:Tdr due to increase in salinity (corresponding to Figure 2.2, point B). A linear regression plotted through data from sites with salinity less than 4% and temperature less than 5 °C but greater than FPD described the increase in ratio of Leu:Tdr due to decrease in temperature (corresponding to Figure 2.2, point C). These equations were used to predict the ratio of Leu:Tdr according to the theoretical model presented in Section 2.2.2. Only ratio data from aerobic waters was used (justified in Section 5.3.2.2). The predicted ratio of Leu:Tdr was plotted against the observed ratio and had a $r^2 = 0.5$ ($n = 48$).

The aspects of the hypothetical model being tested here were: (i) the additive effect of decreasing temperature and increasing salinity; and (ii) the effect of a_w increase due to incubation below FPD. There are many possible problems in relating incorporation of Leu and Tdr; unbalanced growth, non-linear incorporation kinetics, intracellular isotope dilution, non-specific incorporation of isotope, multiphasic uptake, inter- and intra-system variation etc. Nevertheless the ratios for the systems examined in this study conform approximately to predicted values based on an optimised consortia operating at maximal metabolic efficiency. Further observations of the relative activities of adapted consortia to the more extreme salinity increases and/or temperature decreases would help elucidate this relationship.

5.4 Dissolved organic carbon concentrations

The oxylimnion of most of the lakes of the Vestfold Hills and the local marine waters have DOC concentrations of about 1-10 mg C l^{-1}. This is in accordance with global averages for streams and rivers (Collier, 1989). The harshest of the Vestfold Hills lakes, with high salinity, low temperature and/or anoxic conditions, where environmental factors are likely to slow uptake, have much higher DOC concentrations (up to 75 mg C l^{-1}). This simple correlation of reduction in microbial activity with nutrient status bears investigation.

In this study the <1.0 μm fractions of DOC were quantified using wet chemical oxidation methods. It was shown that the <0.2 μm fractions were closely associated with increased bacterial number (Figure 4.25.2). Filtration may rupture fragile planktonic cells and increase DOC (Nagata & Kirchman, 1990). This was manifest as a small increase in DOC concentrations after filtration (Figure 4.24).

DOC concentration in the lakes was uniformly greater than in marine environments. The limited bacterial breakdown of DOC may have been due to the rigours of low temperature and high salinity and was particularly apparent in the anoxylimnion. The high concentrations of refractory DOC in Organic Lake and the anoxylimnion may have been due to a combination of factors, the transformation of labile to refractory DOC through condensation with carbohydrates or other mechanisms (Keil & Kirchman, 1993) and, due to physiological stress, the inability of bacteria to take advantage of nutrient sources which would normally be accessible. The definitions of refractory and labile change with changing conditions. The accumulation of DOC in Organic Lake and the anoxylimnion of Ekho Lake and Fletcher Lake are excellent examples of systems which are under too much stress to take proper advantage of system potential.

Coagulation of DOC on bubbles was shown to be an important mechanism for allowing microbial respiration of otherwise unreactive DOC. Kepkay and Johnson (1989) demonstrated 5-15% of oceanic refractory DOC was metabolised by the general microcommunity after coagulation on bubbles. The lack of wind mixing in ice covered Antarctic lakes may be a critical disadvantage.

Often the microbiota of lake systems is observed to form mucilaginous aggregates where extracellular hydrolytic enzymes would play an important part in hydrolysing refractory DOC. Perhaps the breakdown of this exogenous mucilage system under the harsh conditions of high salt and low temperature in Organic Lake also contributes to the lake's very high concentrations of refractory DOC.

High concentrations of DOC have implications for the ecosystem and may cause; reduced light levels through absorption, low pH caused by high concentrations of organic acids, or absorption of nutrients, trace elements and metals to DOC aggregates reducing their availability to

bacterial processes (Collier *et al.*, 1989). Reduced light levels would inhibit photochemical degradation of DOC. Photochemical degradation is increasingly considered as an important step in making refractory DOC more labile (Mopper *et al.*, 1991).

Biologically labile low molecular weight organic carbon is difficult to detect with wet chemical degradation (Toggweiler, 1992) and is rapidly utilised by bacteria (Ellis-Evans, 1985). Recent evidence suggests that high molecular weight carbon, detectable by the techniques used in this study, may form a large part of the biologically labile carbon fraction (Amon & Brenner, 1994). DOC values from this study were in part indicative of the background of refractory DOC which may not actively influence bacterial activity. The DOC concentrations did not correlate with the peaks in Tdr or Leu incorporation. Rather they were correlated with ratio of Leu:Tdr, a measure of the 'harshness' of the environment.

5.5 Change in total bacterial numbers

Delille *et al.* (1988) found short term variation in bacterial numbers up to almost two orders of magnitude (over 3 days) in Antarctic seawater. This growth rate was confirmed by laboratory experiments and demonstrates the potential activity of Antarctic bacterial communities. Short term variation was stressed by several authors (Albright & McCrae, 1987; Meyer-Reil *et al.*, 1979). Delille *et al.* (1988) found that Antarctic marine bacteria seem to have immediate growth capacity without preliminary reactivation phases. This phenomenon was confirmed by Bjørnsen & Kuparinen (1991).

5.5.1 Bacteriovory and change in bacterial numbers

Bacterial productivity as indicated by Tdr incorporation suggested that total bacterial numbers in the oxylimnion should increase from midwinter to the end of the sampling period. In fact, total bacterial numbers decreased slightly over this time. Bacteriovorous protozoa and phytoplankton may be responsible for this decrease in numbers. During December 1989 and January 1990, John van den Hoff (Australian Antarctic Division, Kingston, Tasmania) quantified the phytoplankton and protozoan population in Ekho Lake. Tucker & Burton (1990) found that peak numbers of the zooplankton in local seawater occurred in March, April and May. These months were not covered by this study.

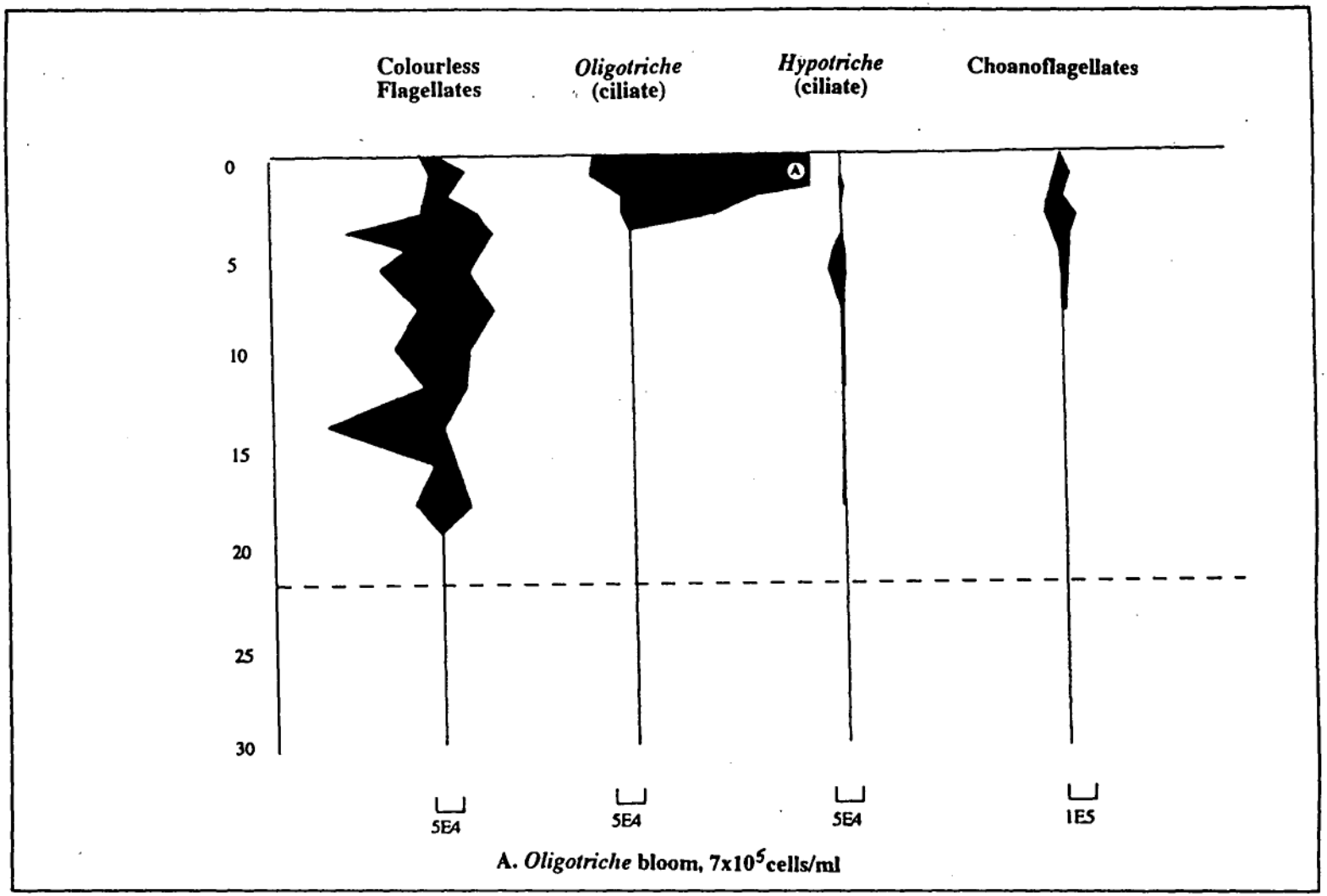
There was a phytoplankton bloom of $2x10^6$ cells ml^{-1} at 20 m in December and a consistent concentration of dinoflagellates of $8x10^5$ cells ml^{-1} at 3 m (Figure 5.2). The bacteriovorous ciliate *Oligotriches* bloomed from 0 to 2 m, $7x10^5$ cells ml^{-1} (Figure 5.4). In Ekho Lake during summer the surface waters were at their most productive, yet bacterial numbers remained relatively constant. Depletion of bacteria is likely to be related to the bloom of the bacteriovorous ciliate *Oligotriches*. In the summer surface waters the rate of Tdr incorporation suggests a clearance rate by the ciliate of at least $3x10^3$ bacteria ml^{-1} h^{-1} or 0.4% of the bacterial population. Other workers have shown that bacteriovorous protozoa are responsible for depletion of bacteria from some freshwater lakes of the Vestfold Hills and Signy Island (J. Laybourn-Parry, Pers. Comm., 1995).

Wilkner & Hagström (1991) found that bacteria have a 'numerical' refuge from bacteriovorous grazing. In estuarine waters bacterial grazing ceased when bacterial numbers were below $7x10^5$ cells ml^{-1}. Bacterial numbers recorded in this study did not fall below $4x10^5$ cells ml^{-1}. This threshold for bacteriovory promotes the coexistence of bacteria and bacteriovours and implies that only actively growing bacterial species are exposed to marked bacteriovory (Wikner & Hagström, 1991)

Solic & Krstulovic (1994) found that an increase in bacterial production was not immediately matched by an equivalent increase in grazing. In Ekho Lake maximum protozoan numbers and maximum bacterial production occur together. This may have been an artefact of the coarse sampling program. Bacteriovorous protozoa stimulate bacterial growth and enhance the turnover of bacterial biomass (Solic & Krstulovic, 1994), so it may be that an increase in protozoa brings about an increase in bacterial productivity rather than the reverse. More detailed study of this area is called for to resolve these questions.

The estimate of bacterial productivity in the hypersaline lakes is less than more temperate waters. Similarly the estimate of maximal grazing rate was substantially less than other studies. Boon and Shiel (1990) estimated the rate of bacterial grazing in an Australian freshwater lake as $<1.5x10^7$ bacteria l^{-1} h^{-1}. They report similar rates in Danish fresh waters. Bloem *et al.* (1989) estimated grazing rates of up to $4.0x10^7$ heterotrophic nanoflagellates l^{-1} h^{-1} in a stratified lake in the Netherlands. The effect of this rate of bacteriovory on uptake

Figure 5.4: Total number of protozoa in the oxylimnion of Ekho Lake in December 1989 (left of median line) and January 1990 (right of median line). Determined by John van den Hoff (Australian Antarctic Division, Kingston, Tasmania).



A. *Oligotriche* bloom, $7x10^5$cells/ml

experiments in this study would not be significant. Over two hours only 1% of the bacterial population would be consumed. This is below the sensitivity of the radioisotope incorporation method.

In this study there was a similar clearance rate about the microaerophilic waters as was attributed to *Oligotriches* sp. or other zooplankton in the oxic waters. Ciliates are found down to the anoxylimnion and would not discriminate between heterotrophic bacteria and cyanobacteria as both are within the particle size for ingestion (Azam *et al., 1983*).

5.5.2 Haline convection and change in bacterial numbers

Albright and McCrae (1987) noted that bacterial concentrations tended to change quickly within the water column and concluded that, "Those data support the observations of other investigators that rapid movement of many biotic and abiotic materials from the surface to deeper waters in coastal seawater columns may occur."

Between October and November 1990 in Organic Lake the water temperature was below -5 °C from 1 to 4 m and the salinity about 16% salts. Total bacterial numbers decreased by $1.5x10^4$ cells ml^{-1} at 2 m but increased by $3.0x10^4$ cells ml^{-1} at 3 to 4 m. November was the start of the summer thaw, decreasing salinity to a depth of 3 m. Changes in bacterial numbers in environments where salinity and temperature seem to preclude such changes are probably the result of haline convection. As mentioned in the literature review, wind induced turbulence mixes the lake waters during summer. Ice cover prevents wind induced mixing for between 8 and 12 months of the year, but progressive growth or reduction of ice cover generates haline convection capable of mixing at least as well (Ferris *et al.*, 1991).

The movement of cells by haline convection cannot be proven in other systems as possible haline convection is accompanied by changes in temperature which may not preclude significant growth. The phenomenon is quite definite in Organic Lake however and on this basis it is likely to occur in other systems. This may be part of the reason Tdr incorporation does not correlate with production estimates based on change in bacterial number.

Many marine bacteria are motile. Similarly, most culturable isolates from the hypersaline lakes of the Vestfold Hills are motile (Dobson, 1988; James, 1989). Some marine bacteria exhibit a chemotactic response when exposed to algal exudates. Bowen *et al.* (1993) demonstrate that chemotactic motile bacteria actively congregate around phytoplanktonic cells to increase their exposure to organic exudate. Given that bacteria can generally follow chemoclines at 1-2 μm s^{-1} (Mitchell *et al.*, 1985) it may be that this effect could explain changes in bacterial population over several metres (1 μm s^{-1} is about 2 m $month^{-1}$).

5.6 Increase in tolerance to salt through uptake of proline

Microorganisms must maintain an internal a_w similar to external solute concentration to prevent osmotic gradients across the cell membrane. Decreased a_w from an increase in external solute concentration can cause dehydration, the effects of which are toxic to most bacteria (Mazur, 1980). Halotolerant bacteria may synthesis or take up compatible solutes. The term 'compatible' indicates compatibility with internal enzymes and metabolic processes. Within the hypersaline lakes of the Vestfold Hills, the ability to resist the effects of increased external solute through the uptake of compatible solutes would confer an advantage. P. Franzmann (Pers. Comm., 1995) has observed that members of the genus *Halomonas* grow more vigorously on AOLPA (120 ‰ salts) (Appendix 1.2.1) on the addition of proline. Wohlfarth *et al.* (1990) showed extensive use of organic compatible solutes for osmoregulation in members of the genus *Halomonas.*

In this study, an experiment to assess the relative growth on increasing salinity media inoculated with Ekho Lake water indicated that on media supplemented with proline, 20% more cultures were able to tolerate increased salinity. This is significant in Ekho Lake waters where the availability of free amino acids is limited to short periods during the season (particularly about the time of algal bloom). *Halomonas* spp., the most common bacteria isolated from the hypersaline lakes of the Vestfold Hills (as indicated by immunofluorescence), are able to use proline and many other amino acids as sole carbon sources (James *et al.*, 1990; Franzmann *et al.*, 1987a). This would further limit the availability of possible osmoprotectants within the water column.

Most halotolerant bacteria protect against osmotic gradients by increasing internal concentrations of organic compatible solutes (Brown *et al.*, 1986; Vreeland *et al.*, 1983). Some of these mechanisms are discussed in detail by Galinski (1993). Compounds which have been implicated in these mechanisms include; betaines, ectoines, glutamic acid, trehalose, polyols and proline (Cummings & Gilmour, 1995; Galinski, 1993; Russell, 1990; Vreeland *et al.*, 1983). Proline was one of eight compounds of algal origin which Mason & Blunden (1989) found to be active as a bacterial osmoprotectant (the other compounds were mainly betaines).

Rosenstock & Simon (1993) studied the abundance and utilisation of dissolved free amino acids (DFAA) in a lake environment. They found that DFAA account for up to 48% of bacterial carbon demand during the phytoplankton spring bloom but less than 5% at other times. Availability and usage covaried i.e. the concentrations of DFAA were uniformly low except at the spring bloom. Glutamic acid, a possible osmoprotectant, was a major component of the DFAA. Münster (1993) supports the view that DFAA are normally only present in threshold concentrations, reaching high concentrations only in association with the growth of algae. Gibson *et al.* (1994) studied the distribution of DFAA in Organic Lake and concluded that concentration in oxylimnion was low but increased in the anoxylimnion were bacterial processes utilising DFAA were reduced by the combination of low temperature, high salinity and lack of oxygen.

The addition of yeast extract to growth media allows some cells to grow at increased salt concentrations as they can accumulate glycine betaine from the yeast extract (Wohlfarth *et al.*, 1990). Wohlfarth *et al.* (1990) postulates that it may be more economical for cells to take up compatible solutes or their precursors than to perform *de novo* synthesis in the cell. In this case the microbiota of Ekho Lake may be limited by lack of suitable osmoprotectants and the energetic cost of de novo synthesis. The possibility is worthy of further investigation. Proline and other DFAA have been shown to be available to heterotrophic bacteria during phytoplankton bloom. The availability of DFAA, sugars, betaines and other potential osmoprotectants at this time may be as important in stimulating bacterial activity as the increased availability of labile carbon.

5.7 Bacterial reduction of DMSO and TMA*N*-O

The anoxylimnion of some of the hypersaline lakes of the Vestfold Hills contain dissolved sulphides and sulphur biology plays an important part in the ecology of these lakes. The reductive capacity of the sulphide helps to maintain the anoxic conditions (Franzmann *et al.*, 1988a). Organic Lake has the highest concentration of dimethyl sulphide (DMS) ever recorded in the natural aquatic environment (Franzmann *et al.*, 1987b). Because of the importance of sulphur biology to the microbial community, heterotrophic bacteria isolated from the lakes of the Vestfold Hills were tested for the ability to reduce DMSO to DMS. The ability to reduce TMA*N*-O to TMA was also tested as TMA*N*-O is another common terminal electron acceptor.

Oxygen supply may be reduced in hypersaline environments. The presence of high concentrations of salt limits gas solubility and reduced mixing in the presence of strong haloclines allows localised depletion of oxygen via respiratory activity. DMSO or TMA*N*-O may be used as alternative terminal electron acceptors to promote growth under reduced oxygen conditions (Oren & Trüper, 1990).

In this study only *D. aquamarina* and *D. venusta* growth was stimulated by the addition of DMSO or TMA*N*-O. Oren & Trüper (1990) investigated the ability of the halophilic archaeobacteria to use DMSO or TMA*N*-O as terminal electron acceptors. They found that under (semi)anaerobic conditions most representatives of the genera *Halobacterium*, *Haloarcula* and *Haloferax* tested were able to reduce DMSO to DMS and TMA*N*-O to TMA; increasing growth rate in most cases. Zinder & Brock (1978) noted that while a wide range of environmental microorganisms could reduce DMSO to DMS, many others could not.

ACAM 379 produced DM_2S in the headspace when grown under anaerobic conditions with methionine as a carbon source, however the greatest production of reduced sulphur compounds was from cultures of the algae *Dunaliella* sp.. Cultures of *Dunaliella* sp. (isolated from Organic Lake) which were in stationary phase produced up to 6 ppm

DM_2S in the headspace and >1 ppm DMS and DM_3S (E. Holdsworth, unpublished data). The presence of *Dunaliella* sp. in Organic Lake was correlated with maximal abundance of DMS (Franzmann *et al.*, 1987b).

Biogenic DMS is a major source of sulphur to the atmosphere and is important in cloud formation (Franzmann *et al.*, 1987b). Most members of the family *Halomonadaceae* did not contribute atmospheric (headspace) reduced sulphur compound under the conditions tested. The *Dunaliella* sp., however, produced DMS, DM_2S and DM_3S during its normal metabolism (E. Holdsworth, unpublished data). A single strain of *H. subglaciescola* (ACAM 2) was shown to produce DMS from methionine and cysteine (Franzmann *et al.*, 1987b), ACAM 379 produced DM_2S when grown in media containing methionine and cysteine.

5.8 Isolation and characterisation of a novel species of the genus *Brevibacterium.*

A new strain was identified from within the water column and sediment of Organic Lake. Culturable bacteria from this lake were generally Gram-negative rods, the new strain was a Gram-positive rod/coccus. It was shown by GC-MS analysis that the strain was able to reduce DMSO to DMS and grow using methionine or cysteine as a sole carbon source. As sulphur biogeochemistry is important to the Organic Lake system and as only 3 other strains from the Vestfold Hills (all *H. meridiana*) were able to utilise methionine and cysteine as sole carbon sources (James *et al.*, 1990), this strain was further characterised.

The novel species of Brevibacterium had a typical coryneform fatty acid profile and clustered with *B. linens* and other *Brevibacterium* spp. and *Arthrobacter* spp. in clusters 'k' and 'l' of subgroup 'D' of the groupings of Bousfield *et al.* (1983). The 16S rRNA sequence for this strain was compared to some closely related organisms (GenBank; Olsen *et al.*, 1991) and grouped closely with *B. linens* and other *Brevibacterium* spp. (Table 4.4). Essential features of the newly defined genus *Brevibacterium* include the occurrence of $MK8(H_2)$ or $MK8(H_2)$ and $MK7(H_2)$ menaquinones and DNA mol% G+C of 60-70 (Fiedler & Bude, 1989). ACAM 379 and ACAM 480 had MK 8(H2) major menaquinone and MK 7(H2) minor menaquinone, no significant ubiquinones (Figure 4.33). ACAM 379 had a mol% G+C between 60-

70, as does ACAM 479 but ACAM 480 had a mol% G+C of 58, outside the current definition of the genus *Brevibacterium*. All strains had a yellow pigmentation which became pink on the addition of 1M NaOH similar to the characteristic test for *B. linens* pigments described in Jones & Keddie (1986).

The coryneform bacteria and isolates from the genera *Arthrobacter* and *Brevibacterium* appear to be common constituents of the Antarctic soil biota (Baker & Smith, 1972; Shivaji *et al.*, 1989; Siebert & Hirsch, 1988; Nedwell & Rutter, 1994). Gram positive strains with a rod-coccus cycle and yellow pigmentation similar to ACAM 379 have been isolated from the soils of the Vestfold Hills (J. Cavanagh, Pers. Comm., 1994) but never before from the water column or sediments of the hypersaline lakes. Based on the characteristics ascribed above the strains ACAM 379, ACAM 479 and ACAM 480 will be attributed to the genus *Brevibacterium* as a novel species once sufficient phenotypic data is collected.

5.8.1 Part description of novel *Brevibacterium* sp..

The novel species of *Brevibacterium* exhibits a rod-coccus cycle during growth. Older cultures are comprised mainly of coccoid cells of 0.6 to 1.2 μm diameter. On transfer to fresh media these give rise to irregular rods of 1.8 to 4.0 μm length. Cells are gram positive but older cultures may decolourise readily. Colonies on solid media are smooth and circular and become yellow with age. Pigment production is not light dependant. The pigment turns 'salmon pink' when treated with a strong base but not when treated with a strong acid.

Optimum growth temperature is between 30 and 32 °C. Growth occurs between -2 °C and 37 °C. Optimum growth is at 0.1% NaCl; growth occurs up to 18% NaCl. Strains grow from pH 4 to 9.

Strains have been deposited with the Australian Collection of Antarctic Microorganisms, University of Tasmania, Sandy Bay, Tasmania as strains ACAM 379, ACAM 479 and ACAM 480.

ACAM 379 has a typical coryneform fatty acid profile (Bousfield *et al.*, 1983) and clusters with *B. linens* and other *Brevibacterium* and *Arthrobacter* spp. in clusters 'k' and 'l' of subgroup 'D' of the groupings

of Bousfield *et al.* (1983). The 16S rRNA sequence for this strain grouped closely with *B. linens* and other *Brevibacterium* spp.. ACAM 379 and ACAM 480 have MK 8(H2) major menaquinone and MK 7(H2) minor menaquinone and no significant ubiquinones. ACAM 379 has a mol% G+C of 62 and ACAM 480 has a mol% G+C of 58.

6 CONCLUSIONS

Analysis of the data collected during this project has provided valuable insight into heterotrophic activity in the oxylimnion of some Antarctic hypersaline lakes. The data emphasises the extreme variability of the seasonal processes in Antarctic aquatic systems.

- Species and strain specific antibacterial antibodies were raised and successfully applied in the study lakes. The *H. meridiana, H. subglaciescola* and *F. gondwanense* serogroups were present in very high proportions and formed discrete populations in aerobic waters amongst the summer biota of Organic Lake and Ekho Lake. In one summer sample, more than 40% of the total bacteria belonged to the genus *Halomonas*. The correlation of the Antarctic summer with maximum serogroup abundance was particularly apparent with *F. gondwanense*.

- The selection of bacteria used in this investigation, isolated on high salinity, high nutrient media at around 10 °C, were representative of the summer hypersaline lake populations in the highly productive areas around the oxycline and in the aerobic waters. Changes in species abundance were not reflected in the total bacterial count, indicating compositional change of the total bacterial population. For the bacteria used in this study, succession was brought about by the onset of summer and the associated environmental changes. The seasonality and discrete locations of these populations is currently the subject of study using species specific fluorescent 16S RNA probes.

- Calibrations of the radioisotope incorporation experiments from this study were similar to values found in other comparable studies. Incubation for 2 hr at *in situ* temperature ± 1.5 °C with either 48 nM of Tdr or 9 nM of Leu was experimentally determined as suitable for all samples. Isotope dilution plots showed a high degree of variation and were non-linear. This finding is in accord with results from Bell (1986), García-Cantizano *et al.* (1994), Pollard & Moriarty (1984) and Riemann *et al.* (1982).

- Though bacterial strains isolated from the study lakes grew optimally at temperatures higher than *in situ* temperature, they were able to habituate to low *in situ* temperature via physiological mechanisms

which were specific to the low temperature. This phenomenon has only recently been emphasised (Russell, 1990; Wiebe *et al.*, 1992). Bacterial productivity *in situ* was determined to be maximal at *in situ* temperature in Ace Lake, Ekho Lake and Organic Lake. This is the first time productivity over a range of temperatures has been reported from the Antarctic environment. This adaptation may be important in redefining the conventional view of bacterial growth under temperature stress.

- Azam *et al.* (1993) indicate that all evidence points to the necessity of specific quantification of bacterial 'hot spots' rather than traditional bulk analyses. Two major areas of increased activity have been identified in this study as being consistent across all study sites; the surface waters around the time of the summer thaw and just above the oxycline at the beginning of the austral spring.

- The rates of Tdr and Leu incorporation in the Antarctic hypersaline lakes and marine site compared well with results from many equivalent studies in Antarctic, Arctic and hypersaline environments. Incorporation rates were generally slow compared to warm water, marine and freshwater studies, productivity being limited by a combination low temperature and high salinity. Trends in seasonal variation were common to all sites and similar to trends determined for non-Antarctic sites from other studies.

- Summer surface water increase in production did not bring about a corresponding increase in bacterial numbers, possibly because there was a commensurate increase in grazers. In Ekho Lake in January at 2 m results suggest a clearance rate by bacteriovory of at least $3x10^3$ bacteria h^{-1} or 0.4% of the bacterial population. As other authors have found, the effect of bacteriovory was self limiting, bacterial populations never fell below $4x10^5$ cells ml^{-1}. Some evidence points to halide convection as an alternative reason for large change in bacterial numbers.

- Within aquatic environments, increase in DFAA, sugars, betaines and other potential osmoprotectants is associated with phytoplankton bloom, at other times these compounds are at threshold concentrations (Münster, 1993). This study found there was a 20% increase in active Ekho Lake bacteria upon the addition of proline to growth media at about the salinity of the mixolimnion of Ekho Lake. The availability of osmoregulatory solutes and their precursors may be as limiting to the

microbiota of the hypersaline lakes as the availability of labile carbon. This possibility should be examined further.

- A hypothetical model for the effects of temperature and salinity on adapted consortia correlated well with DOC concentration, generation time and the ratio of Leu:Tdr. This correlation indicates that the general 'metabolic cost' of survival in increasing salinity or decreasing temperature may be additive and at temperatures below the FPD any further decrease in temperature brings about an increase in a_w experienced by the bacterial cell. In recent times more studies have investigated extreme environments using dual label incorporation techniques which provide data on both Leu and Tdr incorporation. A general model of the effects of temperature and salinity on the ratio of Leu:Tdr in the habituated microbial community is an invaluable aid in the understanding of the nature of these data.

- A novel species of Brevibacterium was partly described in this study from several strains isolated from Organic Lake. Other workers have found bacteria from the genera *Arthrobacter* and *Brevibacterium* in Antarctic soil (Baker & Smith, 1972; Shivaji *et al.*, 1989; Siebert & Hirsch, 1988; Nedwell & Rutter, 1994) and strains similar to the novel species of Brevibacterium have been isolated from the soils of the Vestfold Hills (J. Cavanagh, Pers. Comm., 1994). The novel species of Brevibacterium may be a part of the sulphur cycle within Organic Lake.

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APPENDICES

Appendix 1 Media and solutions list

All media and solutions were sterilised for 15 minutes at 121 °C unless otherwise stated.

1.1 Solutions:

1.1.1 Metals 44 (M 44) (Staley, 1981)

EDTA (Ethylenediaminetetraacetic acid)	2·5	g
$ZnSO_4 \cdot 7H_2O$	10·95	g
$FeSO_4 \cdot 7H_2O$	5·0	g
$MnSO_4 \cdot H_2O$	1·54	g
$CuSO_4 \cdot 5H_2O$	0·392	g
$CoCl_2 \cdot 6H_2O$	0·203	g
$Na_2B_4O_7 \cdot 10H_2O$	0·177	g
Distilled water to	1·0	l

Acidify 500 ml of distilled water with a few drops of H_2SO_4. Dissolve the ingredients and make up to volume with distilled water.

1.1.2 Hutner's modified salts solution (HMSS) (Staley, 1981)

Nitrilotriacetic acid	10·0	g
$MgSO_4 \cdot 7H_2O$	29·7	g
$CaCl_2 \cdot 2H_2O$	3·3	g
$NaMoO_4 \cdot 2H_2O$	12·7	mg
$FeSO_4 \cdot H_2O$	99·0	mg
Metals 44 (1.1.1)	50·0	mL
Distilled water to	1·0	l

Neutralise the nitrilotriacetic acid with KOH. Dissolve the remaining ingredients and adjust the pH to 7·2 with KOH or H_2SO_4. Sterilise and store at 4 °C.

1.1.3 Phosphate supplement (PS) (Mancuso *et al.*, 1991)

K_2HPO_4	2·5	g
KH_2PO_4	2·5	g
Distilled water to	1·0	l

Store at 4 °C.

1.1.4 Artificial Organic Lake vitamin solution (AOLV) (Staley, 1981)

Cyanocobalamin	0·1	mg
Biotin	2·0	mg
Calcium pantothenate	5·0	mg
Folic acid	2·0	mg
Nicotinamide	5·0	mg
Pyridoxine HCl	10·0	mg
Riboflavin	5·0	mg
Thiamine HCl	5·0	mg
Distilled water to	1·0	l

Dissolve and sterilise by filtration (0·2 μm). Store at 4 °C.

1.1.5 Phosphate buffered saline (PBS) (Xu *et al.*, 1984)

NaCl	8.5	g
KH_2PO_4	1.5	g
Na_2HPO_4	9.0	g
Distilled water	1.0	l

Sterilised by filteration through a 0.2 μm mixed cellulose filter before use. Stored at 1 °C in the dark.

1.1.6 Saline-EDTA

NaCl	8.75	g
Na_2 EDTA (Ethylenediaminetetraacetic acid)	37.2	g
Distilled water to final volume	1.0	l

Mix and adjust the pH to 8.0 with NaOH.

1.2 Growth media:

1.2.1 Artificial Organic Lake peptone agar (AOLPA) (Franzmann *et al.*, 1987a)

NaCl	100·0	g
$MgCl_2 \cdot 6H_2O$	5·0	g
$MgSO_4 \cdot 7H_2O$	9·5	g
KCl	5·0	g
$CaCl_2 \cdot 2H_2O$	0·2	g
$(NH_4)_2SO_4$	0·1	g
KNO_3	0·1	g
Peptone (Difco 0118-01-8)	5·0	g
Yeast extract (Difco 0127-02)	1·0	g
Agar (Difco 0140-01)	15·0	g
Distilled water	960·0	ml

Dissolve the ingredients, adjust the pH to 7·0 and add the agar. Boil to dissolve the agar. Sterilise then cool to 50 °C. Aseptically add 20·0 ml of HMSS (Solution 1.1.2), 20·0 mL of PS (Solution 1.1.3) and 1·0 ml of AOLV (Solution 1.1.4).

1.2.2 AOLPA 3%

As in 1.2.1 (above) but with only 30 g NaCl per litre distilled water.

1.2.3 Seawater yeast peptone agar (SWYPA)

Yeast extract (Difco 0127-02)	3·0	g
Peptone (Difco 0118-01-8)	5·0	g
Distilled water	250	ml
Filtered seawater (0·45μm)	750	ml
Agar (Difco 0140-01)	15·0	g

Adjust pH to 7·3. Boil to dissolve agar and sterilise.

1.2.4 Peptone Yeast Vitamin Glucose (PYVG broth)

Hunters mineral salts	20	ml
Peptone	0.25	g
Yeast	0.25	g
Glucose	0.25	g
Distilled water	1000	ml

Double concentrated vitamin solution added aseptically after autoclaving.
18 g l^{-1} agar was added for solid media (PYVG agar).

1.3 Radioisotope incorporation solutions

1.3.1 Stopper solution (modified from Moriarty, 1986)

Gluteraldehyde sol^{n} 25%	100	ml
Thymidine	25	mg

The solution was buffered to pH 8 with sodium bicarbonate and filtered through a 0.2 μm mixed cellulose filter before use. The stopper for leucine incubations contained 25 mg leucine instead of thymidine.

1.3.2 5% trichloroacetic acid

Trichloroacetic acid	1000	g

The trichloroacetic acid was dissolved in 200 ml distilled water giving 800 ml total volume stock solution. Twenty ml of the solution was added to 480 ml of distilled water to give a final TCA concentration of 5%.

1.4 Winkler solutions

Mix solutions and store at 4 °C in the dark.

1.4.1 Manganese chloride solution

$MnCl_2 \cdot 4H_2O$	210	g
Distilled water	500	ml

1.4.2 Potassium iodide solution

KI	75	g
NaOH	250	g
Distilled water	500	ml

1.4.3 Dilute sulphuric acid solution

conc. H_2SO_4	500	ml
Distilled water	500	ml

1.4.4 Sodium thiosulphate solution

$Na_2S_2O_3 \cdot 5H_2O$ stock solution	24.82	g
NaOH	1.0	g
Distilled water	1000	ml

Store in the dark for up to 30 days.

1.4.5 Starch solution

Starch	2	g

Dissolved in 20% NaOH solution then neutralise with conc. HCl.

1.4.6 Potassium iodate standard solution

KIO_3	3.567	g
Distilled water	1000	ml

Store in the dark for up to 30 days.

1.5 Reagents for analysis of menaquinone:

1.5.1 Saponification reagent (P. Holloway, Pers. Comm.)

NaOH	45	g
Methanol (AR)	150	ml
Deionised distilled water	150	ml

Add water and methanol to NaOH pellets. Stir until the pellets have dissolved.

1.5.2 Extraction solvent (P. Holloway, Pers. Comm.)

Hexane (HPLC grade)	200	ml
Methyl-tert Butyl-ether (HPLC grade)	50	ml

1.5.3 Acid solution (P. Holloway, Pers. Comm.)

H_2SO_4 (AR)	20	mg
Distilled water	100	ml

1.5.4 Methylation solvent (P. Holloway, Pers. Comm.)

Chloroform (AR)	10	ml
HCl (AR)	10	ml
Methanol (AR)	100	ml

Appendix 2 List of strains (from Mancuso *et al.*, 1991)

ACAM denotes cultures held at The Australian Collection of Antarctic Microorganisms, University of Tasmania, Hobart, Tasmania, Australia.

ATCC denotes cultures held at The American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, USA.

DSM denotes cultures held in the German Collection of Microorganisms and Cell Cultures Mascheroder Weg 1 b, D-3300 Braunschweig, Federal Republic of Germany.

NCMB denotes cultures held in the National Collection of Marine Bacteria, 23 St Machar Drive, Aberdeen, Scotland AB2 1RY, UK.

UQM denotes cultures held at the Department of Microbiology, University of Queensland, St. Lucia 4067, Queensland. Australia.

Novel species of Brevibacterium .
ACAM 379 *Received from:* E. Holdsworth (Biochemistry). *Isolated from:* Organic Lake, Antarctica (68°27·2'S, 78°12·3'E), sediment 1994. *Culture conditions:* AOLPA 3% (Appendix 1.2.2), aerobic, 25°C.
ACAM 479 *Received from:* E. Holdsworth (Biochemistry). *Isolated from:* Organic Lake, Antarctica (68°27·2'S, 78°12·3'E), sediment 1994. *Culture conditions:* AOLPA 3% (Appendix 1.2.2), aerobic, 25°C.
ACAM 480 *Received from:* P. Hirsch (University of Kiel). *Isolated from:* Organic Lake, Antarctica (68°27·2'S, 78°12·3'E), depth 2 m. *Culture conditions:* AOLPA 3% (Appendix 1.2.2), aerobic, 25°C.
Deleya aesta
ACAM 68T *Received from:* NCMB 1980.
Deleya aquamarina
ACAM 348T *Received from:* DSM 30161.
Deleya cupida
ACAM 343T *Received from:* DSM 4740.
Deleya halophila
ACAM 347T *Received from:* DSM 4770.
Deleya marina
ACAM 344T *Received from:* DSM 4741.
Deleya pacifica
ACAM 345T *Received from:* DSM 4742.

Deleya venusta

ACAM 346T *Received from*: DSM 4743.

Flavobacterium gondwanense

ACAM 44T *Received from*: D. Cameron (ACAM). *Isolated from*: Organic Lake, Antarctica (68°27·2'S, 78°12·3'E), depth 5 m, 1/5/86. *Culture conditions:* SWYA or Marine Agar 2216 (Difco), aerobic, 25°C.

ACAM 46 *Received from:* D. Cameron (ACAM). *Isolated from:* Organic Lake, Antarctica (68°27·2'S, 78°12·3'E), depth 5 m, 1/5/86. *Culture conditions:* SWYA or Marine Agar 2216 (Difco), aerobic, 25°C.

ACAM 49 *Received from:* D. Cameron (ACAM). *Isolated from:* Organic Lake, Antarctica (68°27·2'S, 78°12·3'E), depth 4 m, 1/5/86. *Culture conditions:* SWYA or Marine Agar 2216 (Difco), aerobic, 25°C.

ACAM 56 *Received from:* D. Cameron (ACAM). *Isolated from:* Organic Lake, Antarctica (68°27·2'S, 78°12·3'E), depth 2 m, 1/5/86. *Culture conditions:* SWYA or Marine Agar 2216 (Difco), aerobic, 25°C.

ACAM 62 *Received from:* D. Cameron (ACAM). *Isolated from:* Organic Lake, Antarctica (68°27·2'S, 78°12·3'E), depth 2 m, 1/5/86. *Culture conditions:* SWYA or Marine Agar 2216 (Difco), aerobic, 25°C.

Flavobacterium salegens

ACAM 48 *Received from:* D. Cameron (ACAM). *Isolated from:* Organic Lake, Antarctica (68°27·2'S, 78°12·3'E), depth 4 m, 1/5/86. *Culture conditions:* SWYA or Marine Agar 2216 (Difco), aerobic, 25°C.

ACAM 52 *Received from:* D. Cameron (ACAM). *Isolated from:* Organic Lake, Antarctica (68°27·2'S, 78°12·3'E), depth 3 m, 1/5/86. *Culture conditions:* SWYA or Marine Agar 2216 (Difco), aerobic, 25°C.

ACAM 54 *Received from:* D. Cameron (ACAM). *Isolated from:* Organic Lake, Antarctica (68°27·2'S, 78°12·3'E), depth 3 m, 1/5/86. *Culture conditions:* SWYA or Marine Agar 2216 (Difco), aerobic, 25°C.

Halomonas elongata

ACAM 35T *Received from:* ATCC 33173

Halomonas halmophila

ACAM 71T *Received from:* NCMB 1971.

Halomonas meridiana

ACAM 233 *Received from:* R.C. Garrick (Antarctic Division). *Isolated from:* Organic Lake, Antarctica (68°27·2'S, 78°12·3'E), depth 2 m, 4/87. *Culture conditions:* AOLPA 3% (Appendix 1.2.2), aerobic, 25°C. *Strain at other collections as:* ATCC 49693, UQM 3352.

ACAM 235 *Received from:* R.C. Garrick (Antarctic Division). *Isolated from:* 'Lake Island' Lake, Antarctica (68°33'S, 77°59'E), depth 1 m, 4/87. *Culture conditions:* AOLPA 3% (Appendix 1.2.2), aerobic, 25°C.

ACAM 246T *Received from:* R.C. Garrick (Antarctic Division). *Isolated from:* Burch Lake, Antarctica (68°27'S, 78°16'E), depth 6 m, 4/87. *Culture conditions:* AOLPA 3% (Appendix 1.2.2), aerobic, 25°C. *Strain at other collections as:* ATCC 49692, UQM 3353.

Halomonas subglaciescola

ACAM 12T *Received from:* P.D. Franzmann (Antarctic Division). *Isolated from:* Organic Lake, Antarctica (68°27·2'S, 78°12·3'E), depth 2 m, 24/10/84. *Culture conditions:* AOLPA (Appendix 1.2.1), aerobic, 30°C. *Strain at other collections as:* UQM 2926, ATCC 43668, DSM 4683.

ACAM 21 *Received from:* P.D. Franzmann (Antarctic Division). *Isolated from:* Organic Lake, Antarctica (68°27·2'S, 78°12·3'E), depth 4 m, 24/10/84. *Culture conditions:* AOLPA (Appendix 1.2.1), aerobic, 30°C. *Strain at other collections as:* UQM 2927, ATCC 43669, DSM 4684.

ACAM 221 *Received from:* R.C. Garrick (Antarctic Division). *Isolated from:* Organic Lake, Antarctica (68°27·2'S, 78°12·3'E), depth 4 m, 4/87. *Culture conditions:* AOLPA 3% (Appendix 1.2.2), aerobic, 25°C.

ACAM 222 *Received from:* R.C. Garrick (Antarctic Division). *Isolated from:* Laternula Lake, Antarctica (68°41'S, 77°58'E), surface, 4/87. *Culture conditions:* AOLPA 3% (Appendix 1.2.2), aerobic, 25°C.

ACAM 227 *Received from:* R.C. Garrick (Antarctic Division). *Isolated from:* Organic Lake, Antarctica (68°27·2'S, 78°12·3'E), depth 6 m, 4/87. *Culture conditions:* AOLPA 3% (Appendix 1.2.2), aerobic, 25°C. *Preservation method:* Lyophilisation. *References:* James *et al.*, 1990.

ACAM 229 *Received from:* R.C. Garrick (Antarctic Division). *Isolated from:* Organic Lake, Antarctica (68°27·2'S, 78°12·3'E), depth 5 m, 4/87. *Culture conditions:* AOLPA 3% (Appendix 1.2.2), aerobic, 25°C.

ACAM 230 *Received from:* R.C. Garrick (Antarctic Division). *Isolated from:* Organic Lake, Antarctica (68°27·2'S, 78°12·3'E), depth 1 m, 4/87. *Culture conditions:* AOLPA 3% (Appendix 1.2.2), aerobic, 25°C.

ACAM 233 *Received from:* R.C. Garrick (Antarctic Division). *Isolated from:* Organic Lake, Antarctica (68°27·2'S, 78°12·3'E), depth 6 m, 4/87. *Culture conditions:* AOLPA 3% (Appendix 1.2.2), aerobic, 25°C.

ACAM 251 *Received from:* R.C. Garrick (Antarctic Division). *Isolated from:* Organic Lake, Antarctica (68°27·2'S, 78°12·3'E), depth 3 m, 4/87. *Culture conditions:* AOLPA 3% (Appendix 1.2.2), aerobic, 25°C.

ACAM 255 *Received from:* R.C. Garrick (Antarctic Division). *Isolated from:* Organic Lake, Antarctica (68°27·2'S, 78°12·3'E), depth 6 m, 4/87. *Culture conditions:* AOLPA 3% (Appendix 1.2.2), aerobic, 25°C.

***Pseudomonas* sp.**

ACAM 162 *Received from:* A.J. McGuire (University of Tasmania). *Isolated from:* Burton Lake, Antarctica (68°37·5'S, 78°05'E), depth 10 m, 17/8/84. *Culture conditions:* Marine agar 2216 (Difco), aerobic, 15°C.

Shewanella putrefaciens

ACAM 341 *Received from:* D. Nichols (ACAM). *Isolated from:* Spoiled chicken, Tasmania, Australia. *Culture conditions:* Marine agar 2216 (Difco), aerobic, 15°C.

Appendix 3 Calculation of radioisotope incorporation results (modified from Moriarty, 1990)

Abbreviations are detailed here for ease of reference. Some of these abbreviations have already been defined throughout the thesis, others are specific to this section.

Blanks	DPM of control samples
CLM	chemiluminescence
CPM	counts per minute
DAPI	4,6-diamidino-2-phenylindole
Decay	decay of radioactivity
DPM	disintegrations per minute
ESR	external standard ratio
g	generation time (or doubling time)
Inc	incubation time in minutes
L	mole Leu incorporation ml^{-1} h^{-1}
Leu	L-[3,4,5-^{3}H]leucine
ln	natural log
N	number of bacteria ml^{-1} h^{-1}
N_t	specific growth rate
P	protein synthesis
Radiolysis	demethylation of isotope
SA	specific activity
T	mole Tdr incorporation ml^{-1} h^{-1}
Tdr	[*methyl*-^{3}H]thymidine
Total Bacteria	total direct count in sample (cells ml^{-1})

The series of calculations used to convert CPM data to radioisotope incorporation results are detailed below. Some equations have been emperically derived, others are from literature or supply company sources.

1. CPM were converted to DPM.

(i) The ESR was observed to increase by 0.01 when CLM was greater than 1%.

If CLM>1% then Adjusted ESR = ESR + 0.01

3. The average of adjusted DPM figures was used to calculate T, N and g for Tdr incorporation data or L and P for Leu incorporation data.

(ix) The mole isotope incorporation ml^{-1} h^{-1} (T or L) was calculated from DPM, SA and incubation time (Inc).

$$T\ (mol\ ml^{-1}\ h^{-1}) = (Adjusted\ DPM \div ((SA \times 1000) \times 2.22x10^{12})) \div (10 \times (Inc \div 60))$$

$$L\ (mol\ ml^{-1}\ h^{-1}) = (Adjusted\ DPM \div ((SA \times 1000) \times 2.22x10^{12})) \div (10 \times (Inc \div 60))$$

(x) An estimate of the rate of bacterial division h^{-1} (N) was determined from T with the theoretical conversion factor $5.1x10^{17}$.

$$N\ (cells\ ml^{-1}\ h^{-1}) = (T \times 318 \times 4) \div 2.5x10^{-15}$$

(xi) Generation time was determined as the natural log of 2 divided by the specific growth rate ($N\ N_t^{-1}$). Where N_t was the total bacteria ml^{-1} as measured by vital staining with DAPI.

$$g\ (days) = (ln(2) \div N \div N_t)) \div 24$$

(xii) Protein synthesis (P) was calculated as L multiplied by 1797 (g protein h^{-1}).

$$C\ (g\ ml^{-1}\ h^{-1}) = L \times 1797$$

Appendix 4 Determination of mol% G+C by HPLC

DNA preparation method and analysis was modified from original methods of Mesbah *et al.* (1989). MilliQ water was used for preparation of reagents. All reagents were autoclaved prior to use. Ependorf tubes were used as reaction vessels through-out.

Two colonies, or approximately one loopful of culture, were taken from an agar plate or slope and suspended in 50 μl 0.03 M NaOH by vigorous vortexing. For gram positive or resilient bacteria, 10 μl Lysozyme solution (0.04 g Lysozyme in 1 ml saline-EDTA; Appendix 4.1) was added and the mixture frozen then thawed with liquid nitrogen and hot water then incubated at 60 °C for an hour.

One hundred μl of 2.5% sodium dodecyl sulphate in saline-EDTA and 5 μl of RNAase A (10mg ml^{-1} boiled 1 hour; frozen) were added to the resulting alkaline solution. This was incubated at 55 °C for 30 minutes. Five μl of fresh or frozen Proteinase K (10 mg ml^{-1}; Boehringer) was added then the sample was incubated at 37 °C for 20 minutes.

Forty μl of 5 M NaCl and 200 μl of Chloroform:Isoamyl alcohol (24:1) were added then the sample was gently mixed (wrist action) for 5 minutes and centrifuged at 5000 rpm for 5 minutes in a microfuge. The aqueous phase was carefully collected (losing approximately 1/5th of the material; giving a 150 μl aqueous phase). Care was taken not to include any of the interstitial protein layer.

Three hundred μl 95% Ethanol (-20 °C) was added and the sample incubated at -20 °C for 45 minutes. No sodium acetate was added (Mesbah *et al.* 1989 method indicates the possibility of adding 0.1 M sodium acetate). Sodium acetate is used to help precipitation of small fragment DNA, the sample should contain mostly whole DNA.

The sample was centrifuged at 5000 rpm for 5 minutes then the liquid portion poured off leaving approximately 5 μl with the 'pellet' of DNA material. One hundred μl of 0.1xSSC (Appendix 4.1) was added, the sample gently mixed, then 10 μl of 1 M NaCl was added. Again the sample was gently mixed then 100 μl isopropyl alcohol was added. The mixture was incubated at -20 °C for 45 minutes.

The sample was centrifuged at 5000 rpm for 5 minutes then the liquid portion poured off leaving approximately 5 μl with the 'pellet' of DNA material. Ten μl of 0.01xSSC was added and the mixture stored over night at -20 °C. SSC buffer chelates divalent metal cations and prevents deoxyribonuclease activity. The sample was then dried under vacuum and rehydrated to 10 μl with MilliQ water.

At this stage *E. coli* lambda phage DNA (5 μg in 10 μl of MilliQ distilled water) was processed with the unknown sample as a control and standard.

The sample was boiled for 2 minutes then cooled in an ice bath. Twelve μl of 30 mM sodium acetate buffer, 2 μl of 20 mM $ZnSO_4$ and 2 μl of P1 nuclease (P1 nuclease (340 units per ml) 1 mg ml^{-1} in 30 mM sodium acetate pH 5.3; frozen) was added. The mixture was centrifuged at 2000 rpm for 1 minute and then incubated at 37 °C for 60 minutes. Four μl of alkaline phosphatase (1 μl of fresh 22860 units per ml alkaline phosphatase in 50 μl of glycine buffer; freshly made) was added. The sample was centrifuged at 2000 rpm for 1 minute then incubated for 5.5 hours at 37 °C. Then centrifuged at 10,000 rpm for 5 minutes and frozen at -20 °C overnight.

The following HPLC method was modified from original methods of Mesbah *et al.* (1989) and from C. Dragar (Pers. Comm., 1994) and P. Franzmann (Pers. Comm., 1994).

Twenty mM triethylamine phosphate with 12% methanol was used as a carrier solvent, 500 ml (enough for 12 samples) was freshly made from 20 ml 0.5 M triethylamine phosphate (5.1 ml of Triethylamine in 40 ml water acidified with 50% orthophosphoric acid to pH 5.1 then made up to 100 ml), 60 ml HPLC grade methanol, made up to volume with distilled water then filtered (0.45 μm HV filter).

Between 10 and 20 μl of sample was injected for each 10 minute run, the absorbance measured at 270 nm. Column pressure was approximately 1330 psi at 2 ml per minute flow rate, column dimensions: 100x10 mm, 4 μm packing Nova Pak C18 (Millipore). All samples were run at room temperature.

mol% G+C was calculated from peak integrations (refer to Mesbah *et al.*, 1989 for peak order) using the following formula;

mol% G+C = 100*(xG/(xG+yT))

G and T are the apparent mol fractions of deoxyguanosine and thymidine, x and y are the associated constants (calculated from lambda phage known standard which had a mol% G+C 49.86).

Under some circumstances deoxycytidine or deoxyadenosine may be methylated. Further, the deoxyadenosine peak is toward the end of the chromatogram where accuracy is poor. The mol% G+C was calculated from deoxyguanosine and thymidine peaks only.

4.1 Standard saline citrate buffer (SSC)

Sodium chloride	8.76	g
Trisodium citrate	4.41	g
Distilled water to final volume	1.0	l

Adjust the pH to 7.0 with NaOH. Refrigerate.

Appendix 5 Extraction of bacterial DNA for analysis of 16S rRNA

Bacterial cultures were grown in 500 ml Oxoid nutrient broth (no. 2) to the end of log phase. DNA was extracted by a modified version of Marmur's method (Marmur, 1961), with the addition of a pronase treatment after the sodium dodecyl sulphate step modified from Blackall et al. (1985).

Cells were harvested by centrifugation and rinsed twice by suspension in saline-EDTA (Appendix 5.1) then re-harvesting. The resulting pellet was suspended in 8 ml of saline-EDTA. EDTA inhibits deoxyribonuclease activity which may degrade the DNA.

The suspension was frozen in liquid nitrogen then thawed at 60 °C. Then 0.06 g of lysozyme was added and the suspension shaken vigorously and incubated at 60 °C for an hour.

Then 0.3 g of sodium dodecyl sulphate (sodium lauryl sulphate) and 14 μl of RNAse A (Appendix 5.2) were added. Sodium lauryl sulphate lyses cells, inhibits enzyme activity and denatures some protein. Ribonuclease hydrolyses RNA. The suspension was incubated at 55 °C for 30 minutes. Twenty μl of Pronase (Appendix 5.3) was added and the suspension incubated at 37 °C for 20 minutes. Pronase is a non-specific enzyme which hydrolyses proteins.

Cell lysis was confirmed under the microscope. Then 1.5 g of sodium perchlorate and 8 ml of chloroform : isoamyl alcohol (1:1; Amresco; pH 6.7-8) were added. Sodium perchlorate aids in the disassociation of proteins from nucleic acids. Chloroform denatures proteins, isoamylalcohol reduces foaming, aids the separation and maintains the stability of the layers. The suspension was shaken for 5 minutes then centrifuged at 5000 rpm for 10 minutes. The aqueous phase (top layer) was collected, care was taken not to incorporate any of the interstitial white protein layer.

One volume absolute ethanol (-20 °C) was added to the aqueous phase a little at a time, the precipitating DNA was spooled with a pasteur pipette (with the end melted closed). Cold ethanol causes DNA and RNA to precipitate out of solution as long threads. The spooled DNA was air dried then resuspended in sterile distilled water. The optical density of

the solution was measured at 260 nm (1 OD unit = approximately 50 μg ml^{-1} double stranded DNA). The solution was diluted to about 2 μg ml^{-1} DNA and stored at 4 °C (for no more than 7 days).

5.1 Saline-EDTA

NaCl	8.75	g
Na_2 EDTA (Ethylenediaminetetraacetic acid)	37.2	g
Distilled water to final volume	1.0	l

Mix and adjust the pH to 8.0 with NaOH.

5.2 RNAase

Ribonuclease	2.0	mg
0.15 M NaCl (pH 5.0)	1.0	ml

Heat the solution at 80 °C for 10 minutes. Divide into 14 μl aliquots then freeze at -20 °C.

5.3 Pronase

Pronase	10.0	mg
Tris-EDTA (5.4)	1.0	ml

Incubate the solution for 2 hours at 37 °C then heat at 80 °C for 2 minutes. Divide into 20 μl aliquots and freeze at -20 °C.

5.4 Tris-EDTA

Tris (hydroxymethyl) methylamine	0.121	g
Na_2 EDTA	0.074	g
Distilled water to final volume	100.0	ml

Mix and adjust the pH to 8.1 with NaOH. Refrigerate at 4 °C.

Appendix 6 Analysis of 16S rRNA (modified from Miller, 1994)

6.1 Amplification of 16S rDNA

The bacterial 16S rRNA gene was selectively amplified by polymerase chain reaction (Saiki *et al.*, 1988). Highly conserved sequences at each end of the gene were used as priming sites for synthetic oligonucleotides, primer A and primer H, as described by Edwards *et al.* (1989). Primer A binds the antisense, coding strand of the gene at position 8-27 and primer H binds the sense, non-coding strand at position 1541-1522. These primers facilitate the amplification of a fragment of approximately 1500 nucleotides, encompassing almost the entire gene.

PCR was performed in a DNA Thermal Cycler (Perkin-Elmer Cetus) using reagents from Perkin Elmer Cetus unless otherwise specified. A standard 100 ml reaction contained 10 ng genomic DNA as template, 50 pmol primers A and H, 10 ml 10x PCR buffer II (500 mM KCl, 100 mM Tris-HCl, pH8.3), 200 mM each dNTP (total 0.8 mM dNTPs) (USB), $MgCl_2$ 1.0 mM, 2.5 units *AmpliTaq* ® DNA polymerase and milli-Q water to 100ml. Reactions were covered with 80 ml sterile mineral oil. Ten replicate reactions (=1 ml in total) were regularly done simultaneously to generate template for subsequent sequencing reactions.

The PCR was performed over 35 cycles. Each cycle consisted of denaturation at 94 °C for 1 min, primer annealing at 55 °C for 2 min and extension for 6 min at 72 °C (Edwards *et al.*, 1989). The phenomenon of PCR primers binding non-specifically that has been correlated with protracted extension times (Saiki *et al.*, 1988) was not observed.

6.2 Purification of PCR product

Products of the PCR were run against low molecular weight markers (Amresco) on 1% agarose/TAE gels containing ethidium bromide (0.5 mg ml^{-1}) at 8-10 V cm^{-1} for 30 minutes to 1 hour. DNA was visualised with UV transillumination and the ~1500 bp fragment excised with a scalpel. DNA was removed from the agarose and purified using the GENCLEAN II® Kit (Bio 101 Inc., La Jolla, CA, USA) according to

manufacturers instructions. Yield of PCR product was estimated from A_{260} measurements.

6.3 Sequencing amplified 16S rDNA

Dideoxynucleotide chain termination sequencing (Sanger *et al.*, 1977) adapted for double stranded PCR product (Bachmann *et al.*, 1990), and further modified by Dobson (1993), was used to sequence this major segment of the 16S rRNA gene. The detergent nonidet P40 is added at the annealing step and the annealing process itself involves boiling and snap-freezing the primer/template mix. The sequencing method is described in full as it is a significant departure from customary methods of ds DNA sequencing.

Reagents from the Sequenase® Version 2.0 Kit (USB) were used throughout, including Sequenase® T_7 DNA polymerase. Fragments were labelled with a-^{35}S-dATP (12.5 mCi ml^{-1}).

Synthetic oligonucleotide primers were purchased from Bresatec Ltd.. Primers were used in a such a way that every part of the gene fragment was sequenced at least twice from one or both strands. Where there was ambiguity, an "n" was specified in place of a base in the sequence.

6.3.1 Annealing Reaction

The following reagents were combined in a microcentrifuge tube, boiled for 3 min and dropped immediately into liquid nitrogen.

Sequencing primer (10 pmol ml^{-1})	1.0 ml
DNA template (1 mg ml^{-1})	1.0 ml
R_X buffer	2.0 ml
Nonidet P40 (20%)	1.0 ml
dH_2O	5.0 ml

Tubes were spun briefly to collect condensate and then placed on ice.

6.3.2 Labelling Reaction

Sequenase® T_7 polymerase was diluted 1:8 in "enzyme dilution buffer". To the 10 ml annealed reactants were added in the following order:

0.1 M dithiothreitol	1 ml
Labelling mix (dGTP), undiluted	2 ml
a-^{35}S-dATP (12.5 mCi ml^{-1})	1.5 ml
T_7 DNA polymerase (1:8)	2 ml

The reaction proceeds at room temperature over times ranging from 3 min for sequence close to the primer, to 5 min for sequence further from the primer. When sequence very close to the primer was sought, 1 ml Mn^{2+} buffer from the Sequenase 2.0® kit was added to the labelling reaction.

6.3.3 Termination Reactions

The labelling reaction was terminated by removing 25% of the reaction mix into one of four tubes each containing a dideoxynucleotide corresponding to one of the four bases i.e. ddATP, ddCTP, ddGTP and ddTTP. In this way are the polymerising fragments in each tube terminated at a preordained base. The four termination mixes (2.5 ml) were pre-warmed to 37 °C, 3.5 ml of the labelling reaction added and the reactions incubated for three minutes at 37 °C.

The termination reaction was stopped by the addition of 4 ml formamide-based STOP solution. Reactions were stored at -20 °C for subsequent use, though the addition of Mn^{2+} buffer in the labelling reaction shortens storage time to 2-3 days (USB recommendation).

A second set of reactions was performed simultaneously using the dGTP analogue dITP (deoxy-inosine 5'-triphosphate) and the labelling mix and termination mixes specific to its use (Sequenase 2.0® kit - USB).

6.3.4 Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed using the LKB Macrophor Sequencing System (Pharmacia LKB), including the LKB 2010-001 Macrophor apparatus and the LKB Multitemp II Thermostatic Circulator. Cleaned plates were treated with Bind-Silane

and Repel-Silane (Pharmacia LKB) as recommended by the manufacturer.

Reaction products were separated on a 0.2 mm 6% denaturing (7 M urea) polyacrylamide gel in 1xTBE buffer, at 1800 V, with water circulating through the thermostatic plate at 55 °C. Before loading, the samples were denatured at 90 °C for 3 min.

Four dGTP reactions (terminating at A,C,G and T) were run immediately adjacent to the four dITP reaction derived from the same primer. Equivalent reactions were run for 2 hours and 5 hours side by side; these sequences overlap and could give a combined range of 200-300 bases of readable sequence per primer.

Gels were fixed in 10% acetic acid (v/v) for 20 min while still attached to the non-thermostatic sequencing plate. Gels were air-dried, then exposed to XOMAT-AR film (Eastman Kodak Co.). Autoradiographs were usually developed after 24 hours, though if the isotope was particularly hot, exposure times could be as short as 8-12 hours with no obvious loss of resolution.

6. 4 Taxonomic Analysis

Sequences were read from autoradiographs and recorded using the program Seqspeak 1.0 DNA Sequence Editor by K. Conover (1991, Dalhousie University, Nova Scotia, Canada). Overlap in sequences derived from different primers was identified by eye. Ambiguous nucleotides were recorded as "n" in the sequence.

The 16S rDNA sequence of each species was aligned with the 16S rDNA of the species most closely related. *E. coli* was included as an outlier. Published sequences were taken in a pre-aligned form from the Ribosomal Database (RDB, Olsen *et al.*, 1991). The new sequence was added into the alignment manually using the HOMED (Homologous Sequence Editor) program (Peter Stockwell, 1986 University of Otago, NZ), accessed through ANGIS (Australian National Genomic Information Service - University of Sydney, NSW). The PAUP program (Phylogeny Analysis Using Parsimony) (Smithsonian Institute, 1993) was used to analyse the alignment data.

Appendix 7 Raw lake and seawater data

The following data was interpreted in this thesis and used to generate the figures and tables. Some data has been omitted; full temperature and salinity profiles for the lakes (values every meter), fraction profiles of DOC (<1.0 μm fraction, <0.6 μm fraction, <0.4 μm fraction and unfiltered results) and replicate values and blanks or controls. These data are presented in full in;

James, S.R. 1991. Ecology of Antarctic micro-organisms. Annual report to the Antarctic Division of The Department of Science (Available from Antarctic Division, Kingston, Tasmania).

Table 7.1: Data collected from lake and local seawater sites during the 1990/91 Antarctic season. Abbreviations are as follows;

Lake	Site of sample collection
Depth	Depth (m)
Date	Date
Temp	Temperature (°C)
Sal	Salinity (‰ salts)
Chl	Chlorophyll (g l^{-1})
Oxy	Oxygen (ml l^{-1})
pH	pH
DOC	DOC
Bact	Total bacterial count (cells ml^{-1})
%H.mer	% of *H. meridiana* serogroup positive cells of the total bacterial count (ml^{-1})
%H.sub	% of *H. subglaciescola* serogroup positive cells of the total bacterial count (ml^{-1})
%F.gon	% of *F. gondwanense* serogroup positive cells of the total bacterial count (ml^{-1})
%F.sal	% of *F. salegens* serogroup positive cells of the total bacterial count (ml^{-1})
Tdr‡	Tdr incorporation (mol h^{-1} ml^{-1})
Leu‡	Leu incorporation (mol h^{-1} ml^{-1})
Ratio	Ratio of Leu:Tdr
Sun	Monthly average of sunshine (h day^{-1})

‡ Average result of 2-3 analysis replicates

Lake	Depth	Date	Temp	Sal	Chl	Oxy	pH	DOC	Bact	%H.mer	%H.sub	%F.gon	%F.sal	Tdr	Leu	Ratio	Sun
Ekho	2	19-May-90	-1.4	35	4.4	8.8	7.9	1.8	9.2E+05					0.01			1.3
Ekho	4	19-May-90	8.3	63	4.2	8.5	8.0	1.4	5.4E+05					0.12			1.3
Ekho	6	19-May-90	14.6	68	5.9	9.1	8.1	2.0	7.9E+05					0.16			1.3
Ekho	10	19-May-90	15.2	71	3.8	8.8	8.0	1.9	6.4E+05					0.12			1.3
Ekho	14	19-May-90	17.7	72	7.9	7.9	7.9	2.6						0.05			1.3
Ekho	16	19-May-90	18.8	84	39.1	7.0	7.7	5.0	5.7E+05					0.05			1.3
Ekho	18	19-May-90	18.7	109	4.5	7.8	7.4	5.4	9.9E+05					0.25			1.3
Ekho	20	19-May-90	17.7	125	4.9	6.2	7.4							0.07			1.3
Ekho	22	19-May-90	16.7	138	15.6		7.4	15.8	1.8E+06					0.00			1.3
Ekho	30	19-May-90	15.0	162			7.8	12.6	1.9E+06					0.00			1.3
Ekho	2	15-Jul-90	-1.7	42	0.3	8.1	8.0	2.0	1.8E+06	0.0	0.0	0.0	0.0	0.63			0.4
Ekho	4	15-Jul-90	7.4	60						0.0	0.0	0.0	0.0	0.00			0.4
Ekho	6	15-Jul-90	12.2	69						0.0	0.0	0.0	0.0	0.00			0.4
Ekho	10	15-Jul-90	12.9	71	0.4	9.2	8.0	2.5	1.6E+06	0.0	0.0	0.0	0.0	0.11			0.4
Ekho	14	15-Jul-90	16.6	73	0.3	8.2	8.1	2.8	1.7E+06	0.0	0.0	0.0	0.0	0.00			0.4
Ekho	16	15-Jul-90	18.2	94						0.0	0.0	0.0	0.0	0.00			0.4
Ekho	18	15-Jul-90	18.1	117	0.5	8.1	7.6	5.5	2.5E+06	0.0	0.0	0.0	0.0	0.86			0.4
Ekho	20	15-Jul-90	17.8	131						0.0	0.0	0.0	0.0	0.00			0.4
Ekho	22	15-Jul-90	17.0	142	0.4		7.4	7.5	5.9E+06	0.0	0.0	0.0	0.0	0.00			0.4
Ekho	30	15-Jul-90	15.7	162					8.0E+06	0.0	0.0	0.0	0.0	0.00			0.4
Ekho	2	27-Sep-90	-2.4	29	0.3	8.4	8.0	2.0	6.5E+05	1.3	0.6	0.0	0.0	0.31	2.17	7.0	3.6
Ekho	4	27-Sep-90	4.2	55	0.4	7.3	8.2	2.5	7.9E+05	0.0	1.3	0.0	0.0	0.10	5.75	59.7	3.6
Ekho	6	27-Sep-90	8.1	68	0.3		8.2	2.1	9.0E+05	1.3	0.0	0.0	0.0	0.13	4.87	36.8	3.6
Ekho	10	27-Sep-90	11.0	68	0.3	8.0	8.2	3.1	1.4E+06	0.0	0.0	0.0	0.7	0.36			3.6
Ekho	14	27-Sep-90	12.1	68	0.3	8.2	8.1	4.1	2.1E+06	1.2	1.0	0.4	1.4	0.00			3.6
Ekho	16	27-Sep-90	15.9	81	0.6	8.1	7.9	4.0	1.6E+06	0.3	1.7	0.4	1.4	0.51			3.6
Ekho	18	27-Sep-90	16.7	108		7.7	7.6	18.8	4.2E+06	1.8	0.3		1.3	0.03	2.42	84.2	3.6
Ekho	20	27-Sep-90	16.9	134	1.6	7.6	7.5	20.0	5.3E+06	0.2	0.6	0.4	0.3	0.05	8.30	183.0	3.6
Ekho	22	27-Sep-90	16.6	134			7.5	29.4	1.0E+07	0.7	0.3	0.2	0.1	0.00	8.03		3.6
Ekho	30	27-Sep-90	12.5	160	5.3		7.5	20.0	1.3E+07	0.0	0.0	0.0	0.0	0.00			3.6
Ekho	2	2-Dec-90	0.6	29		8.1	8.2	1.8	9.5E+05	6.0	7.2	0.0	0.0	0.58	12.20	20.9	9.2
Ekho	4	2-Dec-90	6.1	42		8.5	8.0	1.7	9.7E+05	4.6	7.5	0.0	0.0	0.61	7.02	11.5	9.2
Ekho	6	2-Dec-90	11.0	55		10.8	8.2	2.0	1.4E+06		11.0	9.0	0.0	0.00			9.2
Ekho	10	2-Dec-90	11.6	68		8.9	8.1	2.1	9.1E+05	8.0	0.0		0.0	0.62	8.21	13.2	9.2

Lake	Depth	Date	Temp	Sal	Chl	Oxy	pH	DOC	Bact	%H.mer	%H.sub	%F.gon	%F.sal	Tdr	Leu	Ratio	Sun
Ekho	14	2-Dec-90	12.8	68		9.6	8.2	2.3	8.5E+05	10.5	0.0	5.7	0.0	0.05	2.18	48.1	9.2
Ekho	16	2-Dec-90	15.6	81		8.4	8.1	4.9	7.9E+05	23.6	16.4	0.0	0.0	0.48	3.24	6.8	9.2
Ekho	18	2-Dec-90	17.6	81		6.8	7.7	5.2		9.1	3.2	0.2	0.0	0.30	6.27	20.9	9.2
Ekho	20	2-Dec-90	17.3	121		9.4	7.9	12.3	3.6E+06	0.9	0.7	0.0	0.0	0.00	8.56		9.2
Ekho	22	2-Dec-90	16.4	134			7.4	9.8	3.2E+06	3.4	2.7		0.0	0.17	5.76	33.9	9.2
Ekho	30	2-Dec-90	14.5	147			8.0	12.3	4.6E+06	0.0	0.0	0.0	0.0	0.15			9.2
Ekho	2	19-Jan-91	3.1	16		8.6		1.0	7.1E+05	0.9	0.3	1.7	0.0	4.62	7.08		7.4
Ekho	4	19-Jan-91	6.2	16		8.9		1.9	4.8E+05	0.4	0.9	3.1	0.0	1.37	5.26	3.8	7.4
Ekho	6	19-Jan-91	11.9	55		11.9		2.1	6.6E+05	0.0	1.5	2.1	0.0	1.01	10.00	9.9	7.4
Ekho	10	19-Jan-91	11.8	68		10.1		2.1	6.9E+05	0.0	1.2	2.3		0.59	12.20	20.7	7.4
Ekho	14	19-Jan-91	12.4	68		10.2		2.4	8.1E+05	1.3	0.3	3.8	0.0	0.38	10.20	26.7	7.4
Ekho	16	19-Jan-91	15.6	81		9.3		3.7	8.7E+05	3.1	0.7	0.0	0.0	0.32	10.30	32.3	7.4
Ekho	18	19-Jan-91	17.8	108		7.9		21.8		4.3	0.6	0.4	0.0	0.39	23.40	59.7	7.4
Ekho	20	19-Jan-91	17.5	134		7.6		23.5		0.9	1.4	0.2	0.0	0.00	11.10		7.4
Ekho	22	19-Jan-91	16.0	134				12.9	5.0E+06	0.7	1.0	0.0	0.0	0.47	14.20	30.4	7.4
Ekho	30	19-Jan-91	14.6	160				20.0	6.4E+06	0.0	0.0	0.0	0.0	0.00			7.4
Organic	2	17-Jun-90	-7.2	127				39.0		0.0	0.0	0.0	0.0	0.00			0
Organic	3	17-Jun-90	-1.5	171				45.0		0.0	0.0	0.0	0.0	0.00			0
Organic	4	17-Jun-90	0.9	177				42.0		0.0	0.0	0.0	0.0	0.00			0
Organic	5	17-Jun-90	0.6	197				45.0		0.0	0.0	0.0	0.0	0.00			0
Organic	2	17-Aug-90	-10.0	139	2.7	5.7	7.4	32.9	2.7E+06	1.1	0.8	1.2	0.0	0.12	21.80	178.0	2.1
Organic	3	17-Aug-90	-6.3	163	5.5	1.7	7.4	28.8	4.6E+06	0.0	0.5	0.5	0.0	0.02	60.50		2.1
Organic	4	17-Aug-90	-3.7	168	1.0	4.8	7.0	44.7	5.6E+06	0.0	0.4	0.2	0.0	0.00	37.50		2.1
Organic	5	17-Aug-90	-3.5	179	1.8	3.6	7.0	34.7	1.1E+07	0.0	0.0	0.0	0.0	0.00	7.44		2.1
Organic	2	13-Oct-90	-10.4	158	1.0	7.5	7.3		2.0E+07	0.0	1.0	2.1	0.0	0.00	10.80		6.1
Organic	3	13-Oct-90	-10.1	163	1.2	6.2	7.4		5.8E+06	0.0	2.3	6.5	0.2	0.08	3.59	44.5	6.1
Organic	4	13-Oct-90	-5.6	168	2.9	4.1	7.2		1.5E+07	1.9	0.9	2.0	0.4	0.01	6.19	589.0	6.1
Organic	5	13-Oct-90	-3.0	176	0.6	7.6			8.2E+07	0.0	0.3	0.0	0.0	0.23	0.55	2.3	6.1
Organic	2	20-Nov-90	-8.4	147	0.6	9.0	7.3	23.3	5.6E+06	0.0	0.7	10.1	0.0	0.17	23.80	145.0	11.3
Organic	3	20-Nov-90	-7.0	156	1.2	15.9	7.4	24.5	1.3E+07	0.0	2.6	4.5	0.0	0.14	34.60	241.0	11.3
Organic	4	20-Nov-90	-4.7	171	1.5	8.2	7.4	39.0	3.5E+07	0.2	1.6	0.2	0.0	0.23	94.80	421.0	11.3
Organic	5	20-Nov-90	0.5		1.2	4.1	7.3	31.4	1.7E+07	0.1	0.7	0.0	0.0	0.14			11.3
Organic	2	9-Jan-91	7.6	114	0.3	8.0	7.3	38.2	3.6E+06	0.0	0.5	5.8	0.0	1.23	94.20	76.3	7.4
Organic	3	9-Jan-91	5.8	163	1.0	7.7	7.4	30.1	1.0E+07	0.0	3.1	3.3	0.0	0.06			7.4

Lake	Depth	Date	Temp	Sal	Chl	Oxy	pH	DOC	Bact	%H.mer	%H.sub	%F.gon	%F.sal	Tdr	Leu	Ratio	Sun
Organic	4	9-Jan-91	0.6	172	1.1	8.6	7.4	24.2	3.5E+07	0.6	0.9	1.0	0.0	0.00			7.4
Organic	5	9-Jan-91	0.5	171	0.7	7.7	7.3	23.7	3.3E+07	0.0	0.6	0.0	0.0	0.00	52.50		7.4
Fletcher	2	17-Aug-90	-8.9	55	0.2	7.4	7.8		3.7E+06	0.0	0.0	1.9	0.0	0.32	16.40	51.8	2.1
Fletcher	4	17-Aug-90	-7.6	56	0.3	7.7	7.8		5.6E+06	0.0	0.0	0.4	0.0	0.67	11.30	17.0	2.1
Fletcher	6	17-Aug-90	-4.3	71	0.4	7.4	8.1		4.5E+06	0.0	0.0	0.0	0.0	1.31	31.80	24.3	2.1
Fletcher	8.5	17-Aug-90	-3.8	92	62.6		7.3		3.9E+06	0.0	1.1	0.0	0.0	0.00			2.1
Fletcher	11	17-Aug-90	-5.0	126	0.8		7.2							0.00			2.1
Fletcher	2	13-Oct-90	-3.1	60			7.9	1.0	2.6E+06	2.7	0.8	0.0	0.0	0.34	15.20	44.5	6.1
Fletcher	4	13-Oct-90	-3.1	62			8.0		3.0E+06	1.6	1.8	0.0	0.0	0.53	21.30	40.3	6.1
Fletcher	6	13-Oct-90	0.8	68			8.3	2.5	2.9E+06	0.6	0.0	0.0	0.3	0.68	37.00	54.8	6.1
Fletcher	8.5	13-Oct-90	2.9	94			7.2	25.9	6.8E+06	0.0	0.0	0.0	0.0	0.39	9.72	25.3	6.1
Fletcher	11	13-Oct-90		9			7.2	28.3	7.3E+06	0.0	0.0	0.0	0.0	0.24			6.1
Fletcher	2	20-Nov-90	-4.3	68	5.7	9.5	8.0	1.7	3.8E+06	0.5	0.0	0.0	0.0	1.06	30.40	28.6	11.3
Fletcher	4	20-Nov-90	-1.8	68	2.7	12.7	8.3	1.4	3.7E+06	0.0	0.0	0.0	0.0	0.99	18.30	18.5	11.3
Fletcher	6	20-Nov-90	2.7	73	11.7	10.3	8.5	2.5	8.2E+06	0.0	0.0	1.0	0.5	1.48	56.50	38.1	11.3
Fletcher	8.5	20-Nov-90	5.2	87	109.8		7.7	22.0	6.0E+06	0.0	0.0	0.0	0.0	0.00			11.3
Fletcher	11	20-Nov-90	5.3	9	188.3		7.5	46.5						0.00			11.3
Fletcher	2	2-Feb-91	5.3	25	0.2	3.8	7.9	1.4	1.2E+06	0.7	0.1	0.0	0.0	2.66	20.40	7.7	7.4
Fletcher	4	2-Feb-91	6.4	42	0.9	3.1	8.3	1.9	2.2E+06	0.2	0.2	0.0	0.0	2.34	14.90	6.4	7.4
Fletcher	6	2-Feb-91	7.8	48	1.7	2.1	8.4	5.8	3.3E+06	0.0	0.0	0.4	0.0	3.64	32.90	9.0	7.4
Fletcher	8.5	2-Feb-91	6.4	85	40.1		7.7	16.5	5.7E+07	0.0	0.0	0.0	0.0	0.44			7.4
Fletcher	11	2-Feb-91			3.3		7.4	36.5	4.4E+07	0.0	0.0	0.0	0.0	0.00			7.4
Ace	2	6-May-90	-0.3	13	1.4	11.0	8.4	3.9	7.2E+05	0.0	0.0	0.0	0.0	0.00			1.3
Ace	4	6-May-90	-0.2	13	0.6	10.2	8.2	3.9	9.4E+05	0.0	0.0	0.0	0.0	0.12			1.3
Ace	6	6-May-90	6.2	17	1.0	10.2	8.4	5.0	1.5E+06	0.0	0.0	0.0	0.0	0.00			1.3
Ace	11	6-May-90	11.1	31	18.0	7.1	7.4	7.6	5.8E+06	0.0	0.0	0.0	0.0	0.28			1.3
Ace	18	6-May-90	4.9	37	9.9		7.0	18.5	6.3E+06	0.0	0.0	0.0	0.0	0.01			1.3
Ace	2	7-Jul-90	-0.5	19	0.5	7.4	8.3	2.3	5.0E+06	0.0	0.0	0.0	0.0	0.26			0.4
Ace	4	7-Jul-90	0.5	18	0.3	9.1	8.3	2.5	4.0E+06	0.0	0.0	0.0	0.0	0.12			0.4
Ace	6	7-Jul-90	6.6	29	0.4	9.6	8.3	2.8	6.2E+06	0.0	0.0	0.0	0.0	2.06			0.4
Ace	11	7-Jul-90	9.6	34	4.3	5.8	8.1	4.2	4.1E+06	0.0	0.0	0.0	0.0	2.07			0.4
Ace	18	7-Jul-90	4.5	39	1.9		7.2		1.5E+07	0.0	0.0	0.0	0.0	0.30			0.4
Ace	2	21-Sep-90	-0.6	22	0.3	8.5	8.3	3.6	4.9E+06	0.0	0.0	0.0	0.0	0.23	6.85	29.2	3.6
Ace	4	21-Sep-90	-0.3	21	0.4	9.4	8.4	3.6	4.8E+06	0.0	0.0	0.0	0.0	0.78			3.6

Lake	Depth	Date	Temp	Sal	Chl	Oxy	pH	DOC	Bact	%H.mer	%H.sub	%F.gon	%F.sal	Tdr	Leu	Ratio	Sun
Ace	6	21-Sep-90	0.6	23	0.5	7.4	8.4	3.6	5.0E+06	0.0	0.0	0.0	0.0	0.13	43.70	328.0	3.6
Ace	11	21-Sep-90	6.7	41	3.8	10.2	8.4	6.5	9.6E+06	0.0	0.0	0.0	0.0	0.81	19.90	24.5	3.6
Ace	18	21-Sep-90	4.4	43	1.3		7.9	8.7	1.1E+07	0.0	0.0	0.0	0.0	0.00	11.40		3.6
Ace	2	13-Oct-90	-3.1	19			8.3	3.7	1.2E+06	0.0	0.0	0.0	0.0	0.13	12.20	91.1	6.1
Ace	4	13-Oct-90	-3.1	13			8.3	3.7	1.1E+06	0.0	0.0	0.0	0.0	0.00	19.60		6.1
Ace	6	13-Oct-90	-3.1	16			8.3	3.6		0.0	0.0	0.0	0.0	0.14	12.70	93.7	6.1
Ace	11	13-Oct-90	9.8	30			8.2	10.1	7.0E+06	0.0	0.0	0.0	0.0	0.69	15.50	22.5	6.1
Ace	18	13-Oct-90	7.7	30			7.2	13.0	9.8E+06	0.0	0.0	0.0	0.0	0.01			6.1
Ace	2	20-Nov-90	-0.3	12		8.8	8.4	2.6		0.0	0.0	0.0	0.0	0.00	2.36		11.3
Ace	4	20-Nov-90	-0.2	12	0.3	10.2	8.3	2.8		0.0	0.0	0.0	0.0	0.00	3.18		11.3
Ace	6	20-Nov-90	3.0	13	0.3	8.9	8.2	3.1		0.0	0.0	0.0	0.0	0.38	1.33	3.5	11.3
Ace	11	20-Nov-90	11.1	33	27.2	8.5	7.5	8.8		0.0	0.0	0.0	0.0	0.63			11.3
Ace	18	20-Nov-90	8.2	41	2.0		7.2			0.0	0.0	0.0	0.0	1.31	15.30	11.6	11.3
Ace	2	16-Jan-91	2.1	10		7.6	8.3	3.4	1.9E+06	0.0	0.0	0.0	0.0	1.76	22.20	12.6	7.4
Ace	4	16-Jan-91	3.9		0.2	6.9	8.5		2.0E+06	0.0	0.0	0.0	0.0	0.78	11.20	14.4	7.4
Ace	6	16-Jan-91	3.9	16	0.1	5.5	8.3		3.5E+06	0.0	0.0	0.0	0.0	1.24	8.28	6.7	7.4
Ace	11	16-Jan-91		31	4.1		7.5	12.4	5.1E+06	0.0	0.0	0.0	0.0	1.44	5.13	3.6	7.4
Ace	18	16-Jan-91	8.0	42	2.0		7.2	9.6		0.0	0.0	0.0	0.0	0.00			7.4
Seawater	2	17-Jun-90	-1.9	34	0.2	8.2	7.7	0.6	7.2E+05	0.0	0.0	0.0	0.0	0.31			0
Seawater	2	17-Jul-90	-1.8	34	0.5	7.6	7.9	0.3	5.6E+05	0.0	0.0	0.0	0.0	0.49			0.4
Seawater	2	21-Sep-90	-0.8	34	0.3	8.1	7.9	1.6	1.1E+06	0.0	0.0	0.0	0.0	0.17	14.20	82.8	3.6
Seawater	2	30-Sep-90	-0.8	34	1.4	8.4	8.0	1.0	2.4E+06	0.0	0.0	0.0	0.0	0.23	2.18	9.6	3.6
Seawater	2	28-Jan-91	-1.0	34	0.8	10.9	7.9	0.9	1.3E+06	0.0	0.0	0.0	0.0	2.11	10.70	5.0	7.4
Burch	2	18-Aug-90	-9.2	144	0.8	4.1	7.3	13.9	3.8E+05	0.0	0.0	0.0	0.0	0.00			2.1
Burch	4	18-Aug-90	-7.5	167	1.2	4.9	7.4	16.5	7.2E+05	0.0	0.0	0.8	0.0	0.02			2.1
Laturnula	2	21-Aug-90	-5.8	181	0.2	3.9	7.5	14.9	8.1E+05	0.0	0.0	1.8	0.0	0.02			2.1
Laturnula	6	21-Aug-90	1.1	198	1.0	3.8	7.4	15.8	1.1E+06	0.0	0.0	0.6	0.0	0.04			2.1
Cemetary	1	21-Aug-90	-11.3	201	0.2	3.0	7.3		4.0E+06	0.0	0.0	2.0	0.0	0.01			2.1

Time course Incubation (hrs)	1-May-90 Ace L. 4 m 24 nM Tdr	1-May-90 Ace L. 11 m 24 nM Tdr	1-May-90 Ace L. 18 m 24 nM Tdr	16-June-90 Org. L. 5 m 9 nM Leu	8-Jan-91 Org. L. 2 m 48 nM Tdr	8-Jan-91 Org. L. 3 m 48 nM Tdr
0	51	110	26		502	693
0	133	24			150	94
0.5	58	315	90		1186	282
0.5	818	275	26		860	325
1	427	1468	54	7328	3984	422
1	192	538	65	7945	3722	989
2	297	524	33		2636	514
2	319	635	44		1749	453
3	445	1178	48	3588	3182	806
3	454	1275	46	8683	2424	606
4	313	1391	45		2108	2000
4		1096	87		2547	1713
6				2018		
6				4320		
8				7931		
8				4435		
10				7216		
10				4237		

Time course Incubation (hrs)	8-Jan-91 Ekho L. 4 m 48 nM Tdr	8-Jan-91 Ekho L. 16 n 48 nM Tdr	18-May-90 Ekho L. 2 m 24 nM Tdr	18-May-90 Ekho L. 4 m 24 nM Tdr	18-May-90 Ekho L. 16 n 24 nM Tdr	27-Jan-91 SeaWater 48 nM Tdr	16-June-90 SeaWater 9 nM Leu	16-June-90 SeaWater 48 nM Tdr
0	99	313	17	0	9	219	25941	491
0	150	138		26	10	177	27316	1024
0.5	1276	412				1219		
0.5	1216	331				1298		
1	1143	287	23	103	73	2241	18427	86
1	1567	356	20	102	44	2278	23464	
2	2035	488	33	284	81	3429	16990	1294
2	2321	919	45	197	121	3265	21719	710
3	3020	1825	50	228	188	4257		
3	2980	2021	50	329	197	4546		
4	4466	3639	71	441	241	4498		
4	4392	4145	98		247	4850		
6							23890	597
6							8837	
8							7984	115
8								

Table 7.2: Time course data for radioisotope incorporation calibration, results presented as DPM for whole sample (10 ml).

[Isotope] μCi isotope	1-May-90 Ace L. 4 m Tdr 2hrs	1-May-90 Ace L. 11 m Tdr 2hrs	1-May-90 Ace L. 18 m Tdr 2hrs	16-June-90 Org L. 3 m Leu. 2hrs	16-June-90 Org L. 5 m Leu. 2hrs	8-Jan-91 Org L. 3 m Tdr 2hrs
0	9	7	21	83	163	20
0	12	328	35		160	8
1	275	721	40			
1	269	458	21			
5	495	460	31		1128	518
5	290	937	437		655	485
10	297	524	33	13204	3983	578
10	319	635	44		1627	571
20	1002	856	100			514
20	447	1033	110		7118	453
30	661	401	156	45545		
30	2735	1096	982		5411	727
50				62031	6312	
50				50946	12203	

[Isotope] μCi isotope	18-May-90 Ekho L. 2 m Tdr. 2hrs	18-May-90 Ekho L. 4 m Tdr. 2hrs	18-May-90 Ekho L. 16 n Tdr. 2hrs	8-Jan-91 Ekho L. 4 m Tdr. 2hrs	8-Jan-91 Ekho L. 16m Tdr. 2hrs	27-Jan-91 SeaWater Tdr. 2hrs	16-June-90 SeaWater Leu. 2hrs	16-June-90 SeaWater Tdr. 2hrs
0	6	13	15	46	48	25	1420	45
0	11	18	11	24	77	14		22
1	24	98	64					
1	27	254	0					
5	24	97	115	1653	377	3547	2765	227
5	26	132	0	1702	415	3729		
10	33	284	81	2436	702	3391	10790	499
10	45	197	121	2161	994	2931		
20	143	200	162	2035	488			
20	41	240	46	2321	919			
30	55	225	80	2400	1204	3407	36886	719
30	93	196	83	2412	661	4239		904
50							47519	442
50							24087	704

Table 7.3: Isotope concentration data for radioisotope incorporation calibration, results presented as DPM for whole sample (10 ml).

Isotope dilution	1-May-90 Ace L. 4 m	1-May-90 Ace L. 11 m	1-May-90 Ace L. 18 m	8-Jan-91 Org. L. 2 m	8-Jan-91 Org. L. 3 m
µL cold thy.	24 nM Tdr	24 nM Tdr	24 nM Tdr	48 nM Tdr	48 nM Tdr
0	297	524	33	2636	514
0	319	635	44	1749	453
10	440	489	55	1847	777
10	141	388	157	1419	922
20	91	360	46		1383
20	575	316			362
30					2479
30					384
50	69	105	71	1842	745
50	17	520	41	1403	322
100	161	100	41	1509	889
100	381	244	39	1010	232
150	53	84	44	727	591
150	37	119	80		275
Blank	92	67	26	326	394

Isotope dilution	16-June-90 Org. L. 3 m	16-June-90 Org. L. 5 m	16-June-90 SeaWater
µL cold leu.	9 nM Leu	9 nM Leu	9 nM Leu
0			18135
0			13826
10	34047	4918	22897
10	69375	13888	8671
20			21743
20			20610
30	23138	9086	
30		12648	
50	55101	2634	8874
50		5067	7043
100		4352	8312
100		2589	
150	20157	2880	20247
150		3935	22565
Blank	3790	1128	26630

Isotope dilution	18-Jan-91 Ekho L. 4 m	18-Jan-91 Ekho L. 16 n	18-May-90 Ekho L. 2 m	18-May-90 Ekho L. 4 m	18-May-90 Ekho L. 16 n	27-Jan-91 SeaWater	16-June-90 SeaWater
µL cold thy.	48 nM Tdr	48 nM Tdr	24 nM Tdr	24 nM Tdr	24 nM Tdr	24 nM Tdr	24 nM Tdr
0	2035	488	33	284	81	3429	1294
0	2321	919	45	197	121	3265	710
10	1820	479	24	91	54	3165	742
10	1862	704	71	98	34	2667	920
20	1750	492		94	50	3352	
20	2278	677	19	74	42	3794	
30	2342	712		45	41	2926	
30	2167	569		49	40	2703	
50	2253	820	7	52	114	2076	98
50	1620	610	21	96		2287	
100	1268	611	18	82	64	2034	157
100	1371	333	9	33		2686	372
150	1708	366	29	42	38	2615	183
150	1276	582	11	29	25		182
Blank	125	226	17	26	10	198	758

All incubations were for 2 hours.

Table 7.4: Isotope dilution data for radioisotope incorporation calibration, results presented as DPM for whole sample (10 ml).

Incubation Temperature	19-Nov-90 Ace L. 2 m 9 nM Leu	6-July-90 Ace L. 2 m 48 nM Tdr	6-July-90 Ace L. 4 m 48 nM Tdr	6-July-90 Ace L. 6 m 48 nM Tdr	6-July-90 Ace L. 11 m 48 nM Tdr	6-July-90 Ace L. 18 m 48 nM Tdr
-1.5						
-0.5		45.1		97.9	96.9	59.2
0.5	284.1					
1.0				43.0		
1.5						
5.0	315.7		252.6	384.5	178.2	66.6
8.0						
10.0		66.4	78.5		132.7	117.3
15.0						
18.5						

Incubation Temperature (°C)	8-Jan-91 Org. L. 2 m 48 nM Tdr	8-Jan-91 Org. L. 3 m 48 nM Tdr	16-June-90 Org. L. 3 m 9 nM Leu	16-June-90 Org. L. 5 m 9 nM Leu	18-May-90 Ekho L. 6 m 24 nM Tdr
-1.5			7921.8		9.2
-0.5					
0.5	243.2				
1.0					
1.5	382.2	23.1	1394.2	6342.7	
5.0		9.0	10831.4		
8.0	186.6				14.2
10.0			8282.4		
15.0					29.5
18.5	214.9	42.5			

All - 2hrs incubation period

Table 7.5: Incubation temperature data for radioisotope incorporation calibration, results presented as DPM for whole sample (10 ml).